REPRODUCTIVE STIMULATION BY LOW DOSES OF XENOESTROGENS CONTRASTS WITH THE VIEW OF HORMESIS AS AN ADAPTIVE RESPONSE

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Running Title: Hormesis versus xenoestrogenic responses

Summary

We discuss the similarities and differences of two types of effects that occur at low but not high doses of chemicals: hormesis and stimulation by estrogenic endocrine disrupting chemicals or xenoestrogens. While hormesis is a general phenomenon evoked by many compounds, estrogenic stimulation occurs for specific chemicals that disrupt actions of endogenous estrogen. Both types of phenomena can induce an inverted U-shaped dose-response curve, resulting from low-dose stimulation of response, and thus challenge current methods of risk assessment. Hormesis is generally thought to be caused by an overreaction of detoxification mechanisms, which is considered an adaptive response that should protect an organism from subsequent stress. While any stimulatory response may seem beneficial at first sight, in the case of manmade xenoestrogens they are detrimental, and this is demonstrated with examples for low doses of the estrogenic environmental chemicals bisphenol†A and octylphenol, and the estrogenic drug diethylstilbestrol. Adverse effects include oviduct rupture, an enlarged prostate, feminization of males and reduced sperm quality. These maladaptive stimulatory effects divert energy needed for other processes, resulting in reduced fitness. In conclusion, while there are similarities (inverted-U dose-response), there are also differences, adaptive response for hormesis versus maladaptive response for low doses of manmade xenoestrogens, that have been ignored in discussions of hormesis. We propose that the risk posed by low doses of manmade xenoestrogens that show inverted-U responses is underestimated by the current linear-threshold model used in risk assessment, and this is likely to apply to other endocrine disrupting chemicals.

Keywords: hormesis, endocrine disruption, xenoestrogen, bisphenol†A, inverted-U, dynamic energy budget

Introduction

A recent scientific debate concerns the occurrence and evaluation of hormetic responses in toxicology [1-3]. Hormesis is proposed to involve overcompensation that occurs after a toxic insult as homeostasis is reestablished after being disrupted. The stimulatory event is presumably a result of an over-allocation of resources relative to what is needed for repair processes; this is presumed to be adaptive in that it insures that the repair occurs and protects against the possibility of subsequent insult that might occur shortly after the first insult. Because the defining characteristic of hormesis is a stimulation of performance resulting from exposure to low concentrations of chemicals that are toxic at higher doses [4], hormesis might be incorrectly assumed to be based on the same mechanisms involved in the increase in reproductive effort or increase in organ or body size demonstrated by some organisms exposed to low doses of manmade estrogenic endocrine disrupting chemicals (manmade xenoestrogens) that are encountered in the environment.

This paper tries to fuel the current discussion by addressing the distinct similarity (the presence of inverted-U dose-response relationships), as well as differences between responses to low doses of manmade xenoestrogens, which are maladaptive, and hormesis, where responses are viewed as adaptive. We will provide some experimental examples of estrogenic responses, which unequivocally do not fit the assumption of an adaptive response used to describe hormesis [1,2]. Recognition and assessment of an estrogenic response lies at the basis of manmade xenoestrogen risk assessment and is therefore of the utmost importance. In our opinion, it would be a critical mistake to apply the assumptions regarding hormesis to what we believe are clear examples of adverse effects caused by low doses of manmade xenoestrogens and other endocrine disrupting chemicals. This would have an enormous impact on risk assessment, since it would imply that exposure to low doses of toxic chemicals is good for the organism, a view that has led to the suggestion that risk assessment is overprotective and is causing unnecessary fear of exposure to low doses of chemicals [2].

The response systems for estrogen evolved to enable responses to endogenous estrogen. These receptor systems do not have evolved mechanisms that automatically permit discrimination as to whether the stimulation is occurring due to endogenous or exogenous

estrogen. Thus, stimulation does not lead to initiation of repair processes. Estrogens are mitogens and can stimulate cell proliferation at very low doses [5]. The view that hormesis is a tightly regulated slight overcompensation of repair processes, and is thus an adaptive mechanism[†1] has no relevance for estrogenic stimulatory responses initiated by low doses of manmade xenoestrogens within a physiological range of estrogenic activity [5]. This is particularly important in fetuses where homeostatic systems are being established and are not fully functional. We are unaware of any data showing that responses to low doses of manmade xenoestrogens in the environment are beneficial when all responses over the organism°Øs life span are considered. Responses to low doses of manmade xenoestrogens include disruption of the functioning of cells, impaired organ function, disruption of homeostasis, exhaustion of an organism°Øs energy budget, and even an increased mortality rate.

Hormesis *versus* response to low doses of manmade xenoestrogens

For a correct understanding of the debated issue, clear and simple definitions of the hormetic and estrogenic response are required and are provided below.

A hormetic response is the stimulatory response shown by an organism exposed to low concentrations or doses of toxicants, while inhibition of response occurs at much higher doses. Simulation at low doses is most likely caused by an overreaction of an organism°Øs detoxification mechanism [1], which stimulates its entire metabolism, leading to a performance exceeding that of organisms in the control group. Hormesis is attributable to an array of possible working mechanisms and has been found to occur for virtually every endpoint in a wide variety of organisms. It may well have resulted from evolutionary adaptation of organisms to toxic substances present in their environment. Cases of hormesis have been documented for practically all chemicals.

Estrogenic responses can be stimulated at low doses and inhibited at high doses, similar to the dose-response relationship described as hormesis [5]. In contrast to hormesis, estrogenic responses are evoked by specific chemicals. These chemicals exert their effects by either mimicking estradiol (direct effects) or by interfering with the production, metabolism

and transport of estradiol and interfering with estrogen receptors (indirect effects). Manmade chemicals classified as xenoestrogens have to meet certain structural requirements to be able to bind to the estrogen receptor or interfere with a specific component of estrogen biology [5]. Thus, the estrogenic response is a specific effect, such as stimulation of the female reproductive system, that occurs through interaction of a chemical with the classical nuclear estrogen receptor (alpha and beta) or via more recently discovered receptors associated with the rapid induction of second messenger systems. Such interactions may lead to an increased number of eggs or offspring, which is a typical estrogenic response observed in female mollusks exposed to low concentrations of manmade xenoestrogens, such as bisphenol†A (BPA), 4-*tert*-octylphenol and 17α-ethinylestradiol. These chemicals all mimic the natural hormone estradiol [6-8]. A typical response in female mammals is stimulation of the uterus and other reproductive tissues at low but not high doses [9-11]. The situation in males is more complicated, with some reproductive organs being stimulated (prostate) and others inhibited (testes, epididymides and seminal vesicles) in some species [8,12,13].

Examples of effects of manmade xenoestrogens

To illustrate the type of effects and typical inverted U-shaped concentration-response curves that result from exposure to manmade xenoestrogens, three examples are provided here. These are presented since they might be confused as being an adaptive response due to being categorized as hormesis.

1. A 96 h life-cycle test was conducted with the nematode *Caenorhabditis elegans* and 4-*n*-octylphenol (0.1 - 1,000 nM). *C. elegans* was chosen for this assessment, since it possesses an estrogen receptor [14]. A significant increase in the number of juveniles per adult was observed for concentrations up to 100 nM (Fig. 1a). At 1,000 nM, the number of juveniles per adult had returned to control level. Fig. 1b shows the accompanying growth (body length) of the exposed nematodes, which was significantly inhibited at all tested concentrations. For the concentrations 0.1 to 100 nM, the reduced body length may have been the result of allocating the energy to reproduction, rather than to growth; observations that are supported by the Dynamic Energy Budget theory [15]. At 1,000 nM, 4-*n*-octylphenol has

probably reached a toxic level, since it no longer stimulates reproduction but still inhibits growth.

2. In females of the gonochoristic prosobranch snail species *Marisa cornuarietis*, bisphenol†A (BPA) and 4-*tert*-octylphenol induce a complex syndrome of alterations referred to as 'superfemales', even at concentrations as low as 1 _g/L. Affected specimens are characterized by the formation of additional female organs, an enlargement of the accessory pallial sex glands, and a massive stimulation of egg and clutch production (Fig. 2a). This stimulation of egg production during the sexual repose phase of the snails is detrimental to the affected females, since it causes a congestion of clutches in the pallial oviduct, leading to a rupture of the oviduct and ultimately to the female's death. Up to 15.4% of all dissected females exposed to BPA or 4-*tert*-octylphenol exhibited these oviduct ruptures, but the incidence of these malformations was assumed to be much higher. This was deduced from the significant increase in mortality for all BPA and 4-*tert*-octylphenol treatments (Fig. 2b), which is most likely caused by oviduct ruptures. The indication for a female specific mortality in the exposure groups is supported by a slight, although not statistically significant, shift in the sex ratio of surviving animals in favor of males [8]. The reproductive stimulation by BPA in *M. cornuarietis* and the associated mortality are mediated by estrogen receptors, since both effects are fully suppressed in the presence of the anti-estrogens (competitive estrogen receptor antagonists) tamoxifen and ICI 182,780 [16].

3. An inverted-U dose-response relationship for the estrogenic drug diethylstilbestrol (DES) administered to pregnant mice (*Mus musculus domesticus*) on the development of prostate ducts during fetal life and subsequent prostate size and androgen receptor numbers has been shown [17,18] (unpublished observations, Timms and vom Saal). At maternal oral doses of 0.02, 0.2 and 2.0 _g/kg body wt/day, DES stimulated a permanent increase in prostate size in male offspring, while at 20 _g/kg body wt/day no difference from the control was observed, and at 200 _g/kg body wt/day, a significant decrease in prostate size was observed (Fig. 3). Follow-up studies have shown that there are structural differences between the control and 20 _g/kg body wt/day exposed prostate glands. There is also a marked increase in prostate size and hyperplasia of the glandular epithelium at low doses of DES, and a marked

suppression of gland development at the 200 _g/kg body wt/day dose (unpublished observations, Timms and vom Saal). The low versus high dose findings have also been reported for DES by Gupta[†18] in both *in vivo* and *in vitro* experiments. Maternal administration of very low doses of BPA (2.0 to 50 _g/kg body wt/day) also caused an identical permanent stimulation of the prostate and prostate androgen receptors in male mouse offspring, associated with a permanent up-regulation of prostate androgen receptors [13,18] (unpublished observations, Timms and vom Saal). Maternal administration of a low dose of BPA (25 _g/kg body wt/day) also stimulates a similar permanent increase in mammary gland ducts in female mouse offspring [19]. Many other inverted-U dose-response curves for BPA, DES and other endocrine disrupting chemicals have been reported. There are over 100 published studies involving the use of low doses of BPA, including many showing inverted-U dose-response curves. A document containing references to these studies and other information about BPA is available at http://rcp.missouri.edu/endocrinedisruptors/vomsaal/vomsaal.html. None of the reported low dose effects of BPA can be considered beneficial.

It is difficult to imagine anyone proposing that the programming of the prostate to show hyperplasia would ever be desirable, since benign prostate hyperplasia can result in urethral obstruction and ultimately death if untreated in men. Furthermore, in mice, in addition to prostate enlargement, fetal exposure to manmade xenoestrogens such as BPA and DES results in multiple malformations of the urethra, including a marked constriction at the bladder neck (unpublished observations, Timms and vom Saal). The dose range of BPA that produces these effects in mice results in blood levels of unconjugated BPA that are within and even below the range of blood levels measured in human adults and fetuses [20,21]. Thus, adverse effects in mice occur at human exposure levels to BPA and at doses far below the dose predicted to be safe for humans [22].

Discussion

For the untrained observer, confusion between hormetic responses and low-dose effects of endocrine disrupting chemicals, such as environmental estrogens, is quite likely.

However, the mechanism of action of the manmade xenoestrogens described above clearly distinguishes our examples from other types of hormetic responses for which mechanisms are unknown. Endocrine disruptors are defined by their mechanism, namely the type of interference with some aspect of the endocrine system, which includes all intercellular and even autocrine signaling systems. Whereas hormetic responses involve stimulation and are always higher than those of the control (other than perhaps studies of disease frequency), responses to low doses of endocrine disruptors may either be increased or decreased as compared to those of a control group, depending on the specific action of estrogen in the tissue. Therefore, the confusion typically arises in cases of estrogen responses that are stimulatory. Hormesis is regarded as an adaptive and, quite typically, a beneficial phenomenon, because it is considered to result from stimulation of protective mechanisms [2]. In contrast, we are not aware of responses to manmade xenoestrogens encountered in the environment that would be considered beneficial, since they require energy that was not allocated for a particular process in the first place (for tissues where stimulatory effects occur, such as in the oviducts) or they disrupt organ function (for example, the testes). Consider the following cases of estrogenic stimulation:

1. Estrogenic chemical exposure of males in or out of the breeding season may lead to feminization, intersexuality and reduced sperm quality [12,13,23,24].

2. Estrogenic chemical exposure of females out of the breeding season leads to a stimulation of reproduction, which ultimately may cause a rupture of the oviduct as it has been shown for prosobranch snails [8]. Furthermore, this stimulation is likely to cause energy shortages in growth, maintenance and reserves. When exposure occurs out of season, females are unlikely to find a partner to fertilize them, and if, nevertheless, offspring are produced, they will encounter unfavorable circumstances in the outside world (*e.g.* sub-optimal temperatures, lack of food and hiding places) [25,26]. Estrogenic chemical exposure of females in the breeding season may seem the most innocent case, but it could in fact lead to a reduced reproductive performance, which ultimately reduces the number of offspring during the most favorable time for juvenile growth and survival in the environment [27].

3. In mammals, disruption of reproductive processes in offspring can occur following maternal exposure, such as abnormal rates of postnatal growth [28], and changes in adult neuroendocrine and reproductive organ function [29-31]. Developmental exposure to very low doses of manmade xenoestrogens, such as BPA, can lead to early puberty [28], and thus pregnancy during a time in life when fetuses are competing with the growing mother, resulting in a sub-optimal pregnancy, sub-optimal phenotype of offspring, and an increase in mortality [32].

From these examples it should be clear that effects of manmade xenoestrogens cannot be considered to be beneficial to the organism when many outcomes are examined and long-term consequences are considered, unlike what is typically described for hormesis [2]. However, it should be noted that hormetic responses require energy as well, and might therefore induce energy shortages in the same way as manmade xenoestrogens. Also, what may appear to be a short-term advantage of a hormetic response on an isolated system could have adverse consequences over the long term, such as reduced lifespan or increased likelihood of disease of other systems that were not examined. The latter was also acknowledged by Calabrese and Baldwin [1]. Consequently, many supposed examples of hormesis that are considered to be beneficial may not be when all long-term consequences are considered, and this should be considered in future investigations (*e.g.* by studying multiple parameters and long-term effects).

What the two phenomena clearly have in common is the inverted U-shape type of dose-response curve (see Figs. 1a, 2a and 3), which is described as follows: at low concentrations a stimulated performance or response is evident (performance is higher than that of the control), which disappears at higher concentrations (performance is equal to that of the control), and eventually changes to inhibition (performance is lower than that of the control). For xenoestrogens, high dose inhibition can occur due to interference with an increasing number of endocrine-response systems as dose increases (*e.g.* due to binding or cross-talk of a xenoestrogen with other nuclear receptors), activation or inhibition of different genes at different doses [33], and because at increasing concentrations all chemicals, including endogenous hormones, eventually reach toxic levels that will inhibit performance [5].

A critical aspect of the findings presented here is that they demonstrate that low-dose stimulatory effects of manmade xenoestrogens cannot be viewed by regulatory agencies as typically beneficial. In contrast, Calabrese and Baldwin [2] proposed that hormesis should drive a paradigm shift, based on the view that the public has been unnecessarily °Æfrightened°Ø by current assumptions underlying risk assessment. Instead of protecting against low dose exposure, these authors [2] proposed that the fear that there was no safe exposure dose for manmade chemicals should be replaced, based on the recognition that the low-dose beneficial effects of chemicals have been ignored.

It is important to emphasize that we are in complete agreement with the view that the inverted-U functions identified as hormesis [2], and which have also been shown for octylphenol, BPA and DES above, should drive a paradigm shift in risk assessment. This is based on overwhelming evidence from decades of research on hormones and hormone-mimicking chemicals and drugs that: 1. linear extrapolation from experiments using only high doses cannot be used to predict effects at low doses [5]; and 2. at the receptor level there can be no threshold for chemicals that act via the same mechanism as endogenous hormones such as estradiol, since endogenous estradiol is already above the threshold level of activity in the organism [34]. Since these findings falsify the basic assumptions underlying risk assessment for non-carcinogenic chemicals (systemic toxicants), risk assessment as currently conducted using linear extrapolation cannot be considered as a science-based process [35].

While we believe that the findings regarding hormesis and endocrine disruption both show that the linear dose-response model used in current risk assessment has to be abandoned, we draw the opposite conclusion from Calabrese and Baldwin [2]. We propose that with regard to the published findings for endocrine disruptors, the linear-threshold model of risk assessment will dramatically underestimate risk rather than overestimate risk for adverse effects at low doses, which is discussed in detail by Welshons *et al.* [5]. As an example, there are over 30 published studies reporting a wide range of adverse effects at doses of BPA below the current reference dose of 50 _g/kg body wt/day (see http://rcp.missouri.edu/endocrinedisruptors/vomsaal/ vomsaal.html), which the public is

assured is a dose at least 100-fold lower than that which could cause any effects based on the linear-threshold model[†22].

Conclusions

By examining the mode-of-action, our aim has been to clear up confusion between hormesis and responses to low doses of manmade xenoestrogens. The stimulation of performance by manmade xenoestrogens cannot be viewed as the result of an overreacting defense mechanism as in the case of hormesis. Therefore, manmade xenoestrogens should receive special treatment in risk assessment, taking care that even very low concentrations may cause responses deviating from the normal status. Such deviations result in impaired performance, and reduced fitness, since they require an allocation of energy or cause disruption of homeostatic systems that consequently will result in adverse outcomes. These energy shortages and other reductions in fitness may only become apparent when multiple parameters and endpoints, including long-latency outcomes, are determined in a bioassay. We strongly emphasize that the hormesis phenomenon (inverted-U dose-response curves) deserves attention with regard to the current linear-threshold model used in risk assessment, but the view of hormesis as an adaptive response should not be confused with adverse stimulatory responses induced by low doses of manmade xenoestrogens or other endocrine disrupting chemicals.

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Figure legends

Fig. 1. Effects of 4-*n*-octylphenol on nematode (*Caenorhabditis elegans*) reproduction (1a, left) and growth (1b, right). SC†=†solvent control. Symbols are means (*n* = 6, for SC *n* =†11) with standard error. Asterisks denote significant differences from the solvent control (* $p < 0.05$, ** p†<†0.01, *** $p < 0.001$, according to Dunnett's post hoc test following one-way ANOVA).

Fig. 2. Effects of bisphenol†A (BPA) and 4-*tert*-octylphenol (OP) on ramshorn snail (*Marisa cornuarietis*) reproduction (2a, left) and mortality (2b, right). Each exposure group consisted of 240 specimens. SC†=†solvent control. Asterisks in 2b denote significant differences from the solvent control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, according to ¶÷^{2} test) (data from Oehlmann *et al.*[8]).

Fig. 3. Prostate weight (mg) of male mouse offspring (*Mus musculus domesticus*) versus maternally administered diethylstilbestrol (DES) dose (_g/kg body wt/day). Symbols are means with standard error. Asterisks denote significant differences from the control (* $p < 0.05$, according to LSmeans test following one-way ANOVA) (data from vom Saal *et al.*[17]).

Figures

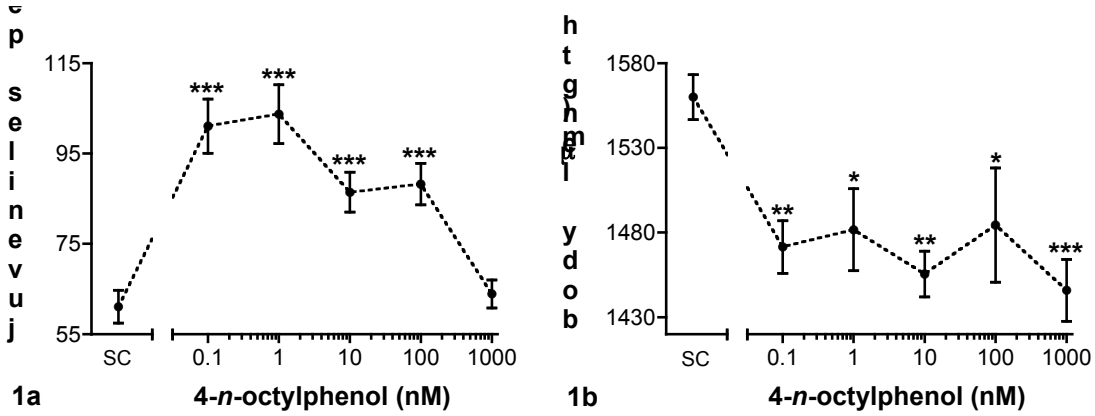

Fig. 1

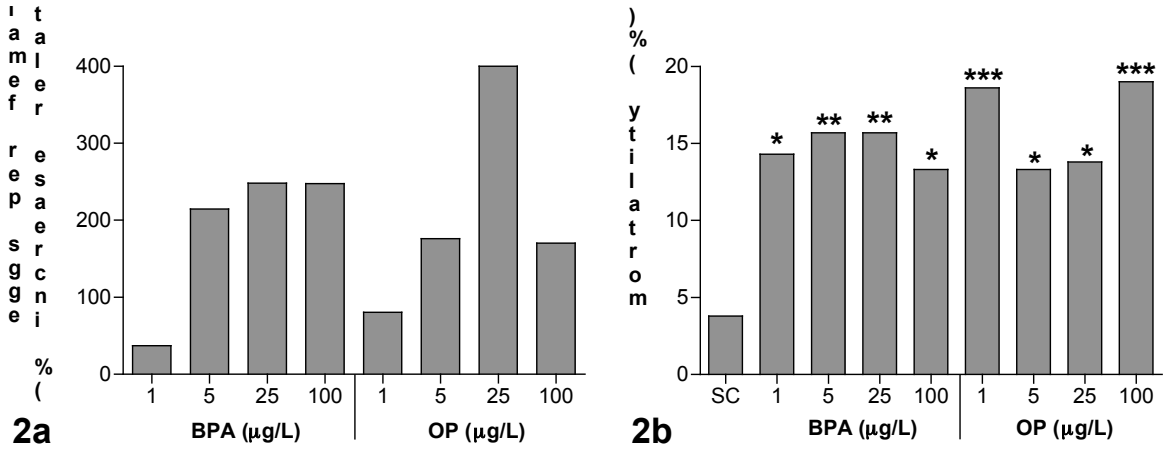

Fig.2

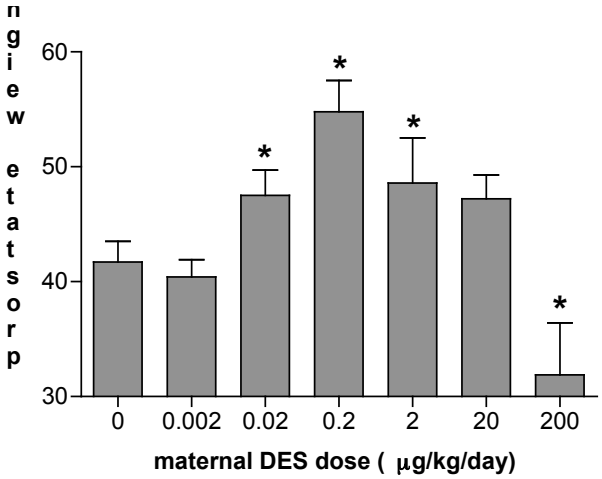

Fig 3