EDITORIAL

Activity of Environmentally Relevant Low Doses of Endocrine Disruptors and the Bisphenol A Controversy: Initial Results Confirmed (44515)

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In this issue of the *Proceedings of the Society for Experimental Biology and Medicine*, Chhanda Gupta (1) reports low-dose effects on prostate development of two endocrine-disrupting chemicals with estrogenic activity, bisphenol A (BPA) and arochlor 1016, a polychlorinated biphenyl (PCB) mixture, as well as an estrogenic drug, diethylstilbestrol (DES), that was included as a positive control. Endocrine disruptors are chemicals that possess unintended hormonal activity or alter normal patterns of hormone effects. This is a recent and controversial area of science, in part because many known or suspected endocrine disruptors are important members of the chemical economy, and the chemical industry is vigorously defending the safety of such chemicals. Additionally, results of some studies have shown that these chemicals can act at very low doses (in the range of human and wildlife environmental exposures to these chemicals) compared with doses normally examined in safety studies (2, 3). Low-dose findings have led to a developing paradigm shift in the way toxicology studies are designed and has increased concern about the safety of such chemicals at ambient environmental levels (4).

In this study, pregnant mice were treated during late gestation with either a high or low dose of DES, a low dose of BPA, or a low dose of PCBs. The low doses of all chemicals increased male anogenital distance (a well-defined developmental estrogenic effect), prostate weight, and prostate androgen receptor ligand binding, and decreased epididymal weight; these effects persisted from shortly after birth through adulthood. The high dose of DES had the opposite effect from the low dose, decreasing male anogenital distance, prostate weight, and androgen receptor binding. Furthermore, strong evidence is presented that the effect of these chemicals is directly on the tissue, as shown by increased prostate weight and androgen receptor binding in fetal prostate cultures treated with low doses of DES, BPA, or PCBs, both in the presence or absence of testosterone.

Importantly, the current study confirms and extends findings from the laboratories of Fred vom Saal and Wade Welshons at the University of Missouri, Columbia. They initially demonstrated that in untreated mice, male fetuses were exposed to estradiol from their female neighbors. Using a microradioimmunoassay, they found increased endogenous serum estradiol concentrations in the male fetuses surrounded by females, relative to levels in fetuses surrounded by no females, and these increased estradiol levels were associated with higher prostate weights and increased prostatic androgen receptors (5). They then showed that treating dams with very low doses of estradiol increased fetal circulating total and free (unbound to serum estrogen binding proteins) estradiol concentrations and increased fetal prostate gland formation, prostate size, and androgen receptor level. A very small increase of 0.1 pg/ml free serum estradiol was sufficient to induce this effect. A more thorough dose-response study showed that doses of DES as

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0037-9727/00/2242-0057$15.00/0

low as 20 ng/kg/day administered during late gestation increased prostate weight and altered prostate morphology (6). Furthermore, as doses increased beyond 200 ng/kg/day, prostate weight decreased, yielding an inverted-U dose-response curve. This group subsequently showed that doses of 2 and 20 μg/kg BPA (a lower potency estrogen) increased prostate weight but decreased epididymal and seminal vesicle weight (7). Thus both the increased prostate weight and the decreased epididymal weight have been confirmed independently by Gupta (1) as well as the opposite effects of low and high doses of DES.

BPA is produced at over 2 billion pounds/year and is found in many products. It is used to coat the inside of cans to prevent exposure of contents to metal, and is found in some dental sealants and in plastics used in automobiles, among many other uses. Of great concern is that BPA is used in the manufacture of many food and beverage containers in addition to cans, such as baby bottles, from which it leaches at an increasing rate as the bottle ages (8, 9). BPA also leaches from cans into foods and is released during hardening of dental sealants (10, 11). Thus, there is human exposure to low doses of BPA from a variety of sources. This exposure is considered safe based on traditional methods of safety evaluation of chemicals. The National Toxicology Program (NTP) conducted a comprehensive study of BPA and found a lowest effect level of 50 mg/kg/day. From these data, they calculated an acceptable exposure value of 50 μg/kg/day (12). The acceptable exposure calculation assumes that a threshold exists. The lowest effect level is divided by a 1,000-fold safety factor to provide a theoretically safe dose. Of great importance is that these presumed safe doses are very rarely tested, because the assumption of a threshold requires that there is no low-dose effect, even when the lowest dose tested still exhibits adverse effects.

The difference between the lowest dose effect levels from the prostate studies and the NTP results is considerable. The lowest effect dose found in fetuses directly tested with low doses (2 μg/kg/day) versus the predicted (but not tested) absence of such low-dose effects based on the NTP high-dose studies (50,000 μg/kg/day) is 25,000-fold. The low-dose effect level (50 μg/kg/day) observed in male mouse fetuses by Gupta (1) is identical to the traditionally calculated safe dose of 50 μg/kg/day, but lower doses were not examined. Furthermore, the 2-μg/kg/day doses of BPA reported in several papers are below this current safe dose (2, 7). This leads to questions regarding which experimental results are right and, of great consequence, how safe BPA really is at the assumed, but not tested, safe exposure of 50 μg/kg/day currently in use.

Why is there such a huge gap in these values? Standard toxicology study design calls first for estimating the dose range for study, which involves calculating the LD_{50} (the dose killing 50% of the animals) or a surrogate, and testing three doses within about two orders of magnitude lower than the LD_{50}. Some of the underlying assumptions in analyzing data generated by such studies are that i) high dose studies predict low dose effects; ii) there is a threshold below which no adverse effects will occur; and iii) the response monotonically increases with dose above the threshold. The low-dose BPA results (2, 7), now confirmed by Gupta (1), demonstrate a nonmonotonic dose-response curve with low doses stimulating an increase in prostate size and high doses decreasing prostate size. Furthermore, we have found no threshold for effects of estradiol on another developmental event, sex-reversal in turtle embryos (3). Thus, the existence of a threshold cannot be assumed. Of considerable interest is the comparison of the lowest effect level between the prostate and turtle results. The single dose lowest effect level for estradiol is 40 ng/kg in the turtle (3), and 20 ng/kg/day (140 ng/kg total dose) in the mouse prostate for DES (6). As estradiol and DES are generally about equipotent, these findings support a low effect level for these estrogens that is up to a million times lower than those often encountered with other types of chemicals. It is clear that if neither a monotonic curve nor a threshold can be assumed, then low-dose effects cannot be predicted from high-dose testing for certain classes of chemicals. One element of the developing paradigm shift is the requirement that chemicals be tested over a much larger dose range than is currently used to collect actual data at low doses rather than assuming low-dose safety (4).

Is there a reasonable biological explanation for low-dose effects of endocrine disruptors? The additivity of an exogenous estrogen dose to circulating estradiol and the lack of a threshold appear key to understanding low-dose effects. The long-standing assumption of a threshold in toxicology is based on the knowledge that organisms possess protective mechanisms (such as repair of damage, replacement of dead cells, etc.) to reduce or eliminate adverse effects. However, when effects produced by endogenous estrogens are experimentally observable, the threshold for effects is already exceeded by the endogenous estradiol because the capacity of the protective mechanisms has been exceeded by the endogenous estradiol. Exposure to a chemical acting through the same mechanism thus may not show a threshold. Furthermore, it has been argued that one would not expect homeostatic regulatory systems that operate in adults to have a similar protective capacity in fetuses in which these very systems are undergoing development (13).

Thus, before exposure to any exogenous estrogen, the endogenous estrogen response is somewhere on the dose-response curve. In this situation, the behavior of the dose-response curve to added estrogen may depend on the nature of the response system. If there are mechanisms operative within the physiological hormone range that alter response rates in a nonmonotonic manner (and these are well known for hormones in general and estrogens specifically (14)), then exposure to exogenous estrogens can result in a nonmonotonic dose-response curve. If such mechanisms do not exist for a particular effect, or are operating at an endogenous estrogen concentration lower than the concentration in the animal, then a monotonic curve that regresses

smoothly to the control value (i.e., does not show a threshold) can result. The low-dose effects then are a consequence of the lack of a threshold; every dose will be active, and detecting activity is only a matter of getting a sufficient response for statistical significance. This differs fundamentally from the threshold model, where the assumption is that a certain dose of an environmental estrogen must be reached before it is possible to cause a statistically significant response.

BPA has a uterotrophic activity about 10^{-4} that of estradiol after development is completed. Thus BPA has been described as a weak estrogen. But even in recent studies conducted in adults, effects below the prior low-effect level of 50 mg/kg/day have been reported (15–18). However, Gupta (1) reports a very low dose direct effect of BPA in cultured fetal prostate of 50 pg/ml medium (≈ 150 fM). BPA may thus be much more potent in fetuses than in adults. This could be due to decreased binding to plasma estrogen-binding proteins (19, 20), increased affinity for ER β, which is highly expressed in the prostate (21), and/or bioaccumulation in pregnant females (but not in nonpregnant adults) (22). It now appears that BPA has a higher estrogenic activity in estrogen-responsive tissues in fetuses (particularly tissues that express ER β) than predicted from the traditional uterotrophic assay conducted by toxicologists after most of development is completed. Thus potency is life-stage–specific as well as tissue-specific.

Of particular importance is that serum estrogen binding proteins (specifically, alphafetoprotein (AFP) in rats and mice, which increase greatly during pregnancy, bind most of circulating estradiol and regulate the bioavailability of endogenous estrogens (19, 20). Chemicals, such as BPA, which fail to bind effectively to these protective proteins, have increased bioavailability, thereby increasing their activity relative to estradiol. Between birth and puberty, AFP levels decrease (23); during the same time the relative potencies of chemicals with low binding affinities for AFP decrease relative to estradiol (24). Of almost 100 naturally occurring and synthetic chemicals evaluated for binding to rat alphafetoprotein, only a few came within even 100-fold of the affinity of estradiol (Branham and Sheehan, unpublished data). This suggests that the increased relative potency of xenoestrogens during development may be a general phenomenon.

It has long been recognized that chemicals that have little or no toxicity in adults can cause malformations and other adverse effects at much lower doses when given during fetal and/or neonatal development (e.g., thalidomide, DES, salicylic acid). This may be due in part to the protective mechanisms not being completely developed (13). Furthermore, developmental effects usually are irreversible. Thus the differences in BPA potency between fetal and adult treatments may be a result of additivity of BPA to endogenous estrogen levels, greater bioavailability of BPA to fetal animals, and greater tissue sensitivity to estrogens during development than during adulthood. Whereas these explanations may help explain the current 25,000-fold difference between effect levels in adult safety studies and the developmental studies, they hold no explanatory power for the failure to replicate the developmental effects.

Industry responded to the initial results with BPA by attempting to repeat the key findings. Two recently published studies (25, 26) failed to find increased prostate weight with fetal exposure to low doses of BPA or DES. The dose used by Gupta (1) fell within the range of doses used in these two studies. Cagen *et al.* (25) asserted that BPA should not be considered a developmental toxicant. Neither group found low-dose effects with a single dose of DES, which presumably was used as a positive control for BPA. The confirmation by Gupta (1) of the positive BPA study (2), and the additional finding of a possible mechanism (an estrogen-induced increase in AR levels, leading to a greater prostate response than in controls) now constitutes a new challenge, not only to industry but to our basic understanding of the ability of current test guidelines to predict safety. Replication and confirmation are crucial elements in science. When contradictory data exist, it is important to evaluate not only the studies in dispute, but also the knowledge and experience of the investigators and any other studies that can cast light on the issue. The two positive studies were conducted by investigators with a long history of experience in the development of the male reproductive tract and extensive knowledge of the literature and of specialized techniques. The two studies that showed similar positive results (1, 2) are bolstered by their biological plausibility, consistent evidence from other related studies, and the experience of the investigators. Finally, there are numerous ways that complex experiments can fail, but often only a few ways that they can succeed.

The challenge now to the scientific and regulatory community is to increase the number and types of low-dose studies of endocrine disruptors, not only to put to rest the BPA issue, but also to determine the extent to which low-dose effects are elicited with other endocrine disruptors. Further investigation of low-dose effects of endocrine disruptors is crucial, as the human population is exposed to tens of thousands of chemicals in the environment, and the weight of the evidence suggests that some of these may cause adverse effects at ambient concentrations.

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