Strain Differences in Vaginal Responses to the Xenoestrogen Bisphenol A

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Bisphenol A (BPA) is the monomer component of polycarbonate plastics and epoxy resins; human exposure derives from leachate in foodstuffs packaged in certain plastics or from epoxy-based dental appliances. BPA stimulates prolactin secretion in Fischer 344 (F344) rats but not in Sprague-Dawley (S-D) rats. The present studies were performed to determine if another classic estrogen target tissue, the rat vagina, responds to BPA in a strain-specific manner. In F344 rats BPA increased DNA synthesis in vaginal epithelium with a median effective dose (ED_{50}) of 37.5 mg/kg body weight; DNA synthesis was not stimulated in S-D rats by any dose tested. Clearance of ^{3}H-BPA from blood followed the same time course in both strains of rats, with a half-life of 90 min. Scatchard analysis of [^{3}H]estradiol binding showed no strain differences in concentration or affinity of the vaginal estrogen receptor. BPA increased the level of mRNA for the immediate early gene, c-*fos*, with similar dose–response curves in both rat strains. Thus, F344 and S-D rats exhibit differences in sensitivity to BPA at the level of cell proliferation in the vaginal epithelium. However, metabolic clearance of BPA and the early events that lead to the proliferative response, receptor-ligand interaction and induction of immediate early genes, show no strain differences. These observations suggest that differences in intermediate effects must account for the difference in sensitivity of the proliferative response to the xenoestrogen. Furthermore, these results point to the need for caution in choosing a suitable end point and animal model when seeking to test the estrogenic effects of xenobiotics. *Key words*: bisphenol A, cell proliferation, c-*fos*, dose response, rat, vagina, xenoestrogen. *Environ Health Perspect* 108:243–247 (2000). [Online 8 February 2000] *http://ehpnet1.niehs.nih.gov/docs/2000/108p243-247long/abstract.html*

Bisphenol A (BPA) is the monomer component of polycarbonate plastics. It behaves as a weak estrogen in classic bioassays, including cellular proliferation and cornification of the vaginal epithelium (*1,2*) and pituitary prolactin secretion (*3*) in the ovariectomized rat, and it produces estrogen-like effects in rat mammary gland (*4*) and developing mouse prostate (*5*). Nearly 2 billion pounds of BPA are manufactured annually in the United States (*6*). Although it is almost entirely in its polymerized form, the BPA monomer finds its way into foodstuffs as a leachate of plastic packaging (*7,8*). Also, humans can be exposed to BPA from certain dental appliances (*9,10*). Because of its estrogenic activity, there is concern over human exposure to BPA.

When assessing the biologic activity of a putative estrogenic compound in animal model systems, it is important to consider species and strain differences. We previously found that BPA increased blood prolactin levels in Fischer 344 (F344) but not Sprague-Dawley (S-D) rats (*3*). Such a finding is congruent with the earlier observation that estrogens stimulate growth of the pituitary through replication of the prolactin-secreting cells in F344 rats but not in outbred rat strains (*11–13*). We also found that the uterine epithelium of F344 rats but not that of S-D rats exhibited a hypertrophic response to BPA administered in low doses (*2*). These observations raise the question of whether there is a general difference between strains in responsiveness of estrogen target tissues to BPA.

The vaginal epithelium is a classic target tissue for studying the estrogenicity of a compound. Estrogens induce proliferation of the cells in the basal layer, thereby producing a multilayered, thick stratified epithelium; with continued estrogen stimulation the superficial layer of cells becomes keratinized (*14*). More recently, it was shown that the immediate early genes, c-*fos* and c-*jun*, are induced by estrogen in target tissues (*15–18*). The induction of these genes is believed to play a key role in cell proliferation (*19,20*). In the present study, we determined the potency of BPA to induce immediate early gene expression and DNA synthetic activity in the vaginal epithelium of F344 and S-D rats.

Materials and Methods

Animals. All procedures performed on animals followed the NIH *Guide for the Care and Use of Laboratory Animals* (*21*) and were approved by the Indiana University Animal Care and Use Committee. F344 and S-D rats were supplied at 6–8 weeks of age by Harlan Sprague-Dawley (Indianapolis, IN). One week after arrival, animals were ovariectomized under ketamine anesthesia; they were subjected to experimental manipulation 3 weeks later. BPA (Aldrich Chemical Co., Milwaukee, WI) and 17β-estradiol (E_2; Sigma Chemical Company, St. Louis, MO) were dissolved in ethanol and diluted in sesame oil. Compounds were injected intraperitoneally (ip) in 50 µL of solution. We injected BPA at 0.2–150 mg/kg body weight (bw) and E_2 at 0.02–2.0 µg/kg bw.

Bromodeoxyuridine immunostaining. Animals were injected with bromodeoxyuridine (BrdU; 100 mg/kg bw in 500 µL saline, ip) and killed 1 hr later. Tissue taken from treated animals was fixed in ethanol–chloroform–acetic acid (60:30:10, vol/vol/vol) at room temperature for 24 hr. The tissue was embedded in paraffin and cross-sections (6 µm) were prepared. After deparaffinization, slides were treated with 0.6% H_2O_2 in methanol for 30 min and then subjected to acid hydrolysis by incubation in 1 N HCl for 8 min, followed by 0.0125 M borate buffer (pH 8.5) for 15 min. Sections were incubated in 5% horse serum to block nonspecific immunostaining and then incubated in anti-BrdU antibody (Becton Dickson, San Jose, CA) overnight, followed by incubation in a biotinylated secondary antibody and avidin-biotinylated peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogen. We counted the BrdU-labeled epithelial cells and determined the length of the underlying basement membrane using image analysis software (IPLab Spectrum; Signal Analytics, Vienna, VA). Results were expressed as the number of labeled cells per millimeter; using several sections from each animal, we counted labeled cells along a sufficient length of basement membrane to include > 1,000 basal epithelial cells.

BPA clearance. To determine the relative rate of clearance of radiolabeled BPA from blood after a single bolus intravenous injection, we anesthetized two animals (150 g bw) of each strain and injected a tail vein

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with 25 µCi (2.9 µg/kg bw) ^{3}H-BPA (13 Ci/mmol; Moravek Biochemicals, Brea, CA) in 250 µL balanced salt solution containing 5% bovine calf serum. Blood was collected into heparinized capillary tubes from a separate tail vein at intervals between 5 and 120 min. After centrifugation, 50 µL plasma was added to a scintillation vial and counted. The half-life of the radiolabeled compound was determined from samples taken between 30 and 120 min.

Estrogen receptor assays. Vaginas from ovariectomized adult rats were homogenized and centrifuged to prepare a cytosol, as described previously (*22*). Aliquots of the cytosol were incubated with increasing concentrations of [^{3}H]estradiol (Amersham, Arlington Heights, IL) with or without a 100-fold molar excess of diethylstilbestrol (Sigma Chemical Company). Bound and free steroid were separated by the dextran-coated charcoal method (*22*).

RNA isolation and RNase protection assays for c-fos. Total RNA was isolated from vagina by homogenizing the tissues in Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNase protection assays (RPAs) were performed on 5 µg RNA from each specimen using a kit (RPA II; Ambion, Austin, TX) according to the manufacturer's instructions. We derived RNA probes from c-*fos* cDNA as described earlier by Nephew et al. (*23*); the plasmid was linearized with the restriction enzyme *Nco*I. Antisense riboprobes were generated from linearized templates using the MAXIscript kit (Ambion), T_7 RNA polymerase, and ^{32}P-uridine triphosphate (New England Nuclear, Boston, MA) according to the kit instructions. Cyclophilin mRNA levels were determined using riboprobe derived from the cDNA template pTRI-cyclophilin (Ambion). After incubation of RNA samples with the radiolabeled riboprobe and RNase, the protected riboprobe was separated on a polyacrylamide gel (6%). The gel was dried and exposed to X-ray film (Kodak X-Omat, Sigma Chemical Company). The X-ray film was analyzed on a GS-670 Imaging Densitometer (BioRad, Hercules, CA). The mRNA levels for c-*fos* were normalized against the level of cyclophilin mRNA by dividing the optical density (OD) of the autoradiographic band for c-*fos* by the OD of the corresponding cyclophilin band of each specimen.

Statistical analysis. The number of BrdU-labeled cells per millimeter or the mRNA levels (arbitrary units of the OD ratios) were analyzed by analysis of variance (ANOVA) across all doses of BPA or E_2, comparing the mean at each dose against the untreated control by Fisher's protected least significant difference (PLSD) test.

Results

In an earlier study (*2*), we determined that the maximum DNA synthesis in vaginal epithelium occurred 20 hr after estrogenic stimulation; therefore, we used this time to determine the dose–response effects of BPA in F344 and S-D rats. The DNA synthetic response to BPA in the vaginal epithelium was dramatically different between the two strains of rats. In F344 rats there was a clear dose response, with a statistically significant increase occurring at 37.5 mg/kg; this dose also corresponds to the approximate median effective dose (ED_{50}) for this effect (Figure 1). However, in S-D rats there was no effect of BPA on vaginal DNA synthesis. In contrast to BPA, there was no strain difference in the vaginal DNA synthetic response to E_2. Although there was a slight difference in the magnitude of the maximal effect (the S-D rats had a higher number of labeled cells), the ED_{50} for E_2 was approximately the same in each strain (Figure 2). It was also apparent that the maximal effect of BPA in F344 rats was approximately one-third of the maximal effect of E_2 (compare Figures 1 and 2).

A simple explanation for the strain difference might be a difference in the rate of metabolic clearance of injected BPA. To test this, animals received an intravenous injection of ^{3}H-BPA and the amount of radioactivity remaining in the blood was determined at various times thereafter, without regard to the proportions of parent compound or metabolite present. Disappearance of radiolabeled BPA was biphasic. After a rapid distribution phase, there was a sustained loss of compound exhibiting a half-life of approximately 90 min for each strain (Figure 3). In two of the animals, approximately one-half of the injected dose of radiolabel was found in the urine that remained in the urinary bladder at the end of 2 hr. Thus, there is a rapid clearance of BPA from the blood, and there does not appear to be any strain difference in this parameter.

The difference in sensitivity to BPA might be explained by a difference in the tissue

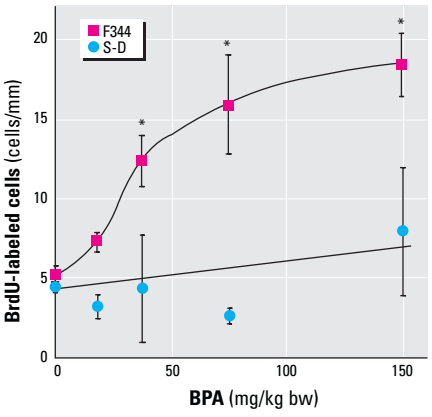

Figure 1. DNA synthetic response to BPA in vaginal epithelia of F344 and S-D rats. Ovariectomized adult rats were treated with BPA at the indicated doses and with BrdU 20 hr later. Values shown are mean ± SE; n = 3–5. F344 data from Steinmetz et al. (*2*).
*$p < 0.05$ vs. control.

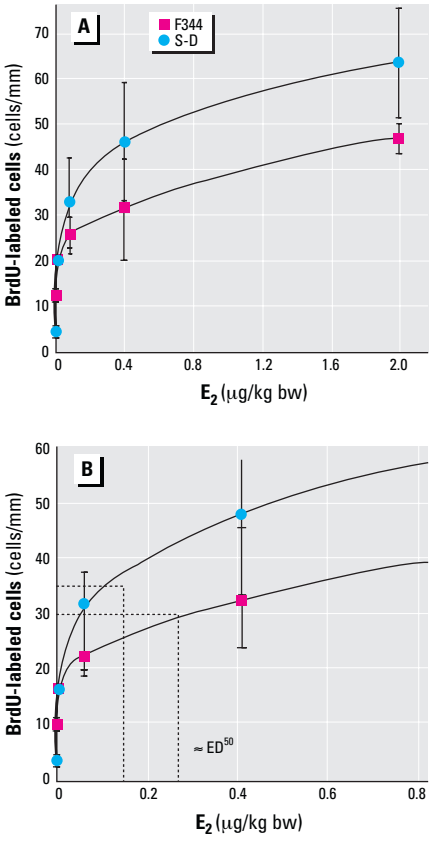

Figure 2. DNA synthetic response to E_2 in vaginal epithelia of F344 and S-D rats. (*A*) DNA synthetic dose response. (*B*) Expanded view of the low end of the dose–response curve to allow estimation of the ED_{50} values. Values shown are mean ± SE; n = 3–5. Ovariectomized rats were treated with the indicated dose of E_2 and with BrdU 20 hr later.

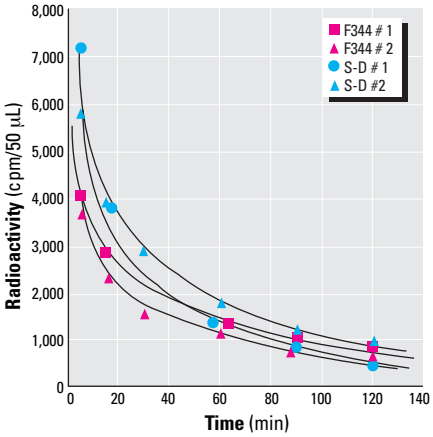

Figure 3. Plasma levels of radioactivity following intravenous injection of ^{3}H-BPA in two animals of each strain. Details are provided in "Materials and Methods."

concentration of estrogen receptor (ER) between rats. To examine this possibility, we analyzed the ER content of vaginal tissue taken from each strain. Scatchard analysis of [^{3}H]estradiol binding showed that there were no differences in concentration or affinity of receptors between the strains (Figure 4). Combining data from four such analyses, ER isolated from F344 rats had a K_d of 0.13 ± 0.023 nM (mean ± SE) and a maximum binding capacity of 266 ± 52.4 fmol/mg protein (mean ± SE), whereas ER from S-D rats had a K_d of 0.14 ± 0.018 nM and a binding capacity of 288 ± 68.4 fmol/mg protein.

The DNA synthetic response occurs several hours after the initial stimulus; this response probably occurs as the result of a cascade of events that are initiated by direct induction of gene transcription. The immediate early gene c-*fos* is part of the primary response to estrogenic stimuli. In a preliminary experiment, animals were treated with 150 mg/kg bw BPA or vehicle and killed at 2, 3, 6, and 24 hr. The RPA of vaginal RNA for c-*fos* showed that BPA induced maximum steady-state levels at 2 hr (Figure 5A); the time course was similar in both strains. We then performed BPA dose–response studies in which animals were sacrificed 2 hr after administration of the xenobiotic. Stimulation of c-*fos* expression by BPA showed no strain difference; both strains of rats responded equally well, exhibiting ED_{50} values at 37.5 mg/kg (Figure 5B).

Discussion

Although the xenobiotic BPA is considered a weak estrogen, there is mounting evidence that it can in fact elicit full estrogenic activity in some bioassay systems. Treatment of immature or adult ovariectomized rats with BPA produced little or no increase in uterine weight (*2,24,25*) and it can partially inhibit the uterotrophic effect of estradiol (*24*), responses typical of a partial estrogen. However, a 3-day treatment of ovariectomized rats with BPA caused the vaginal epithelium to become fully keratinized (*1,2*); this is the response of a full agonist. Also, prolactin secretion was increased to the same extent by estradiol or BPA, but pituitary growth was not induced (*3*). Induction of c-*fos* expression by BPA reached a magnitude, and followed a time-course, that would be expected for a natural estrogen (*15–18*). In a preliminary study, we also found that BPA induced expression of c-*jun* in both strains of rats, with steady-state levels reaching maximum at 2–3 hr after treatment (data not shown). Likewise, the time-course of the DNA synthetic response in the F344 vagina following a single injection of BPA is similar to that produced by natural estrogens (*26,27*). In studies on estrogenic effects in non-reproductive tissues, Dodge et al. (*25*) found that BPA lowered cholesterol levels but did not protect against bone loss in ovariectomized rats. Thus, depending on the end point and the animal model under study, BPA may be considered a partial or a full estrogen agonist *in vivo*. On the other hand, BPA produces full estrogenic effects in breast cancer cells *in vitro* (*9*). Perhaps the recently coined terminology "selective ER modulator" (SERM) (*28*) is a more appropriate characterization of the activity of BPA.

The ED_{50} values for BPA effects, as determined in this study, were approximately 40 mg/kg bw. It is unlikely that such a high dose would be encountered in a single environmental exposure. However, the apparent *in vivo* potency of BPA is dependent on the route of administration and dosing schedule. When BPA was applied by subcutaneous insertion of a continuous release capsule that delivered approximately 300 µg/kg bw/day, a full estrogenic response was produced in the vaginal epithelium of F344 rats (*2*). Thus, the biologic potency of this compound may depend on dosage, dosing schedule, and route of administration.

Biologic potency also depends on the metabolic clearance rate of a compound, and this may differ between the two strains of rats. To address this, we investigated the pharmacokinetics of a bolus injection of BPA. Knaak and Sullivan (*29*) showed that over the first 24 hr after BPA was administered orally to rats, > 80% of the administered material was excreted as a glucuronide in the urine or eliminated as free compound and a hydroxylated metabolite in the feces. Also, 8 days after administration, no BPA was detected in the animals (*29*). In the present study, circulating BPA had a half-life of approximately 90 min. Although this simple analysis did not determine whether the radiolabel remaining in the blood was authentic starting material, it nonetheless indicates that there is a rapid clearance of BPA from the blood. Furthermore, the rate of clearance was similar in F344 and S-D rats, indicating that strain-specific metabolic clearance does not play a role in establishing the difference in the biologic responses. Further studies are required to determine additional pharmacokinetic parameters under the condition of a continuous administration regimen.

Our results show that the F344 inbred rat strain is more sensitive to BPA induction of DNA synthesis in vaginal epithelium than the outbred S-D rat strain. Furthermore, this strain difference appears to be specific to the weakly estrogenic compound BPA, as there was no difference between strains in sensitivity to E_2 stimulation. Yet, the initial events that lead to the

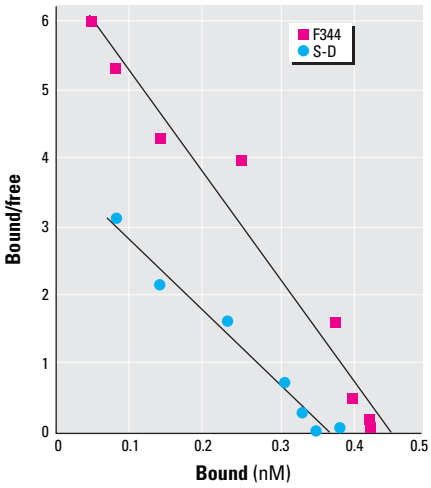

Figure 4. Scatchard analysis of vaginal estrogen receptor. Cytosol was prepared from vaginas of F344 and S-D rats. A standard saturation analysis of binding activity for [^{3}H]estradiol was performed using the dextran-coated charcoal method. A typical Scatchard plot from one of four such assays is shown.

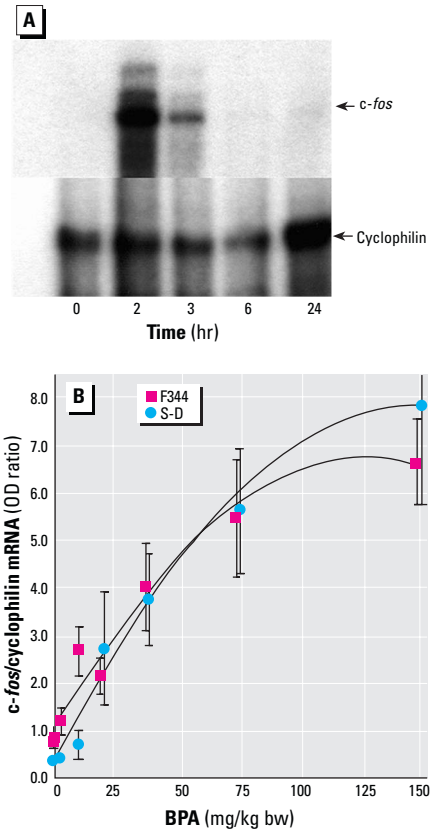

Figure 5. RPA determinations of c-*fos* mRNA steady state. (*A*) An autoradiogram of the RPA bands for c-*fos* and cyclophilin at various times after BPA treatment in F344 rats. (*B*) Steady-state levels of c-*fos* were determined 2 hr after BPA treatment in F344 and S-D rats. The intensity (OD) of the *c-fos* band was normalized against the OD of the corresponding cyclophilin band. Values shown are mean ± SE; $n = 4$.

DNA synthetic response, ER binding and immediate early gene transcription, show no strain differences that account for the difference in sensitivity to BPA. Although BPA induced expression of immediate early genes in S-D rats, this expression was not sufficient to produce the proliferative response; this is similar to a single dose of either of the weak estrogens estriol or 16α-E_2, which was able to induce expression of immediate early genes but did not induce the growth response in rat uterus (*30,31*). In a preliminary study, we found that three daily injections of estriol produced a uterine growth response and that this treatment was more effective in F344 rats than in S-D rats (*32*).

Our observations suggest that the strain difference is due to a delayed, or intermediate, effect rather than a primary response mechanism. Lanahan et al. (*33*) described a set of "delayed early genes" whose expression is induced by growth stimuli, but only after several hours. Using a short-acting estrogen, 16α-E_2, Stack and Gorski (*34*) showed that stimulation of DNA synthesis in the rat uterus is positively correlated to the rate of protein synthesis at 12 hr after an injection of estrogen. Cheng and Pollard (*35*) showed that uterine expression of c-*ras*H and ornithine decarboxylase increased 6–12 hr after estradiol treatment. Dean and Sanders (*36*) suggested that there are two classes of genes which respond to estrogenic stimuli in a delayed manner: the secondary response genes that are dependent on the products of the early primary response genes for their stimulation, and the delayed primary response genes that are dependent on a direct interaction of steroid receptor with the gene's promoter and concomitant enhancement by a product of the early primary response gene. It may be that the delayed response genes stimulated by E_2 in both S-D and F344 rats are not induced by BPA in the S-D rat but are induced by BPA in the F344 rat; this possibility requires further investigation.

Strain differences in response to estrogenic stimuli have been previously demonstrated. Gorski and co-workers (*11–13*) found that the potent estrogens diethylstilbestrol or E_2 induce an overgrowth of lactotropes in the pituitary glands of F344 rats but not in those of outbred strains of rats. Recently, Spearow et al. (*37*) found dramatic strain differences in the susceptibility of mice to estradiol-induced disruption of testicular development. Others have shown differences in the efficacy of E_2 in stimulation of uterine DNA synthesis between strains of mice (*38,39*). Our data extend these observations to the vaginal response in rats and point out strain differences in sensitivity to a weak estrogen, BPA. This type of difference must be taken into account when utilizing the classic vaginal response model for assessment of estrogenic activity of test compounds.

The genetic parameters that are responsible for strain differences in estrogen sensitivity or efficacy are largely unknown. However, Roper et al. (*40*) recently reported that several genetic quantitative trait loci may be partly responsible for the varied efficacy of E_2 to induce uterine growth in different strains of mice. Similarly, Wendell and Gorski (*13*) identified five quantitative trait loci that genetically account for more than half of the difference between estrogen-induced growth of pituitary glands in F344 rats and Brown Norway rats, but these chromosomal loci do not correspond to those that account for strain differences in uterotrophic responses in mice (*40*). At present, although a number of genes located within the quantitative trait loci are known, the specific genes responsible for these strain differences have not been identified (*40*). There were no strain differences in estrogenic stimulation of several oncogenes or angiogenic factors in the pituitary gland; therefore, these genes are unlikely to mediate this effect (*41*). Similarly, we found that the immediate early genes c-*fos* and c-*jun* had no apparent role in establishing the strain difference to BPA stimulation in the vagina. Also, we have found that the ERs of each strain of rats have the same characteristic binding affinity and tissue concentration, ruling out alterations at this level as the simple explanation. As mentioned above, it may be that a later event, such as modulation of an intermediate gene, accounts for the strain differences. Identification of the genetic traits responsible for strain differences in sensitivity to the action of weak estrogens in rodents may yield valuable insight into the causes of varied susceptibilities to xenoestrogen action in humans.

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