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 (ED50) of 37.5 mg/kg body weight; DNA synthesis was not stimulated in S-D rats by any dose tested. Clearance of [3H]-BPA from blood followed the same time course in both strains of rats, with a half-life of 90 min. Scatchard analysis of [3H]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 xenoestrogens. 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.ornl.gov/docs/2000/108p243-247/long/abstract.html

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) 3H-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 [3H]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 Neor. Antisense riboprobes were generated from linearized templates using the MAXIscript kit (Ambion), T7 RNA polymerase, and 32P-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-cyclophillin (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-Ormat, 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 E2, 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 μg/kg; this dose also corresponds to the approximate median effective dose (ED50) 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 E2. 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 ED50 for E2 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 E2 (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 3H-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 radiolabeled 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
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 dynamic 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 \(\mu\)g/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 weak estrogenic compound BPA, as there was no difference between strains in sensitivity to \(E_2\) stimulation. Yet, the initial events that lead to the
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 estradiol or 16α-E2, 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 estradiol 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-E2, 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-raf1 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 are those 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 E2 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 E2 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 E2 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 a genetic quantitative trait loci may be partly responsible for the varied efficacy of E2 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 uterotropic 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 insights into the causes of varied susceptibilities to xenostrogens in action in humans.

### References and Notes

1. Dodds EC, Lawson W. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137:966 (1936).
2. Steinmetz R, Mitchner NA, Grant A, Allen DL, Bigsby RM, Ben-Jonathan N. The xenostrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology 139:2741–2747 (1998).
3. Steinmetz R, Brown NG, Allen DL, Bigsby RM, Ben-Jonathan N. The environmental estrogen bisphenol A stimulates prolactin release in vitro and in vivo. Endocrinology 138:1786–1789 (1997).
4. Colerangle JB, Roy D. Profound effects of the weak environmental estrogen-like chemical bisphenol A on the growth of the mammary gland of female rats. J Steroid Biochem Mol Biol 10:153–161 (1997).
5. Nagel SC, von Saal FS, Thayer KA, Dhar MG, Bouchier M, Welhons WW. Relative binding affinity- serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivities of the environmental bisphenol A and octylphenol. Environ Health Perspect 105:70–76 (1997).
6. Ben-Jonathan N, Steinmetz R. Xenoestrogens: the emerging story of bisphenol A. Trends Endocrinol Metab 9:124–128 (1998).
7. Bratton JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenostrogen release from lacquer food coatings in food cans. Environ Health Perspect 113:608–612 (1995).
8. Sharman M, Honeybone CA, Jickells SM, Castle L. Detection of residues of the epoxy adhesive component bisphenol A diglycidyl ether (BADGE) in microwave susceptible and its migration into food. Food Addit Contam 12:779–787 (1995).
9. Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Ferrante A, Pedraza V, Soto AM, Sonnenschein C. Estradiol activity of resin-based composites and sealants used in dentistry. Environ Health Perspect 104:298–305 (1996).
10. Sonnenschein C, Soto AM. An updated review of environmental estrogen and androgen mimics and antagonists. J Steroid Biochem Mol Biol 69:143–150 (1999).
11. Wolkund J, Wertz N, Gorski J. A comparison of estrogen action on uterine and pituitary growth and prolactin synthesis in F344 and Holtzman rats. Endocrinology 139:1802–1807 (1998).
12. Wolkund JA, Gorski J. Genetic differences in estrogen-induced deoxyribonucleic acid synthesis in the rat pituitary: correlations with pituitary tumor susceptibility. Endocrinology 136:2773–2778 (1997).
13. Wendell DL, Gorski J. Quantitative trait loci for estrogen-dependent pituitary tumor growth in the rat. Mamm Genome 8:232–235 (1997).
14. Dorfman RI. Standard methods adopted by official organizations. In: Methods in Hormone Research, Vol II (Dorfman RL, ed). New York: Academic Press, 1962:707–729.
15. Loose-Mitchell DS, DiPetta C, Stancel GM. Estrogen regulation of c-fos messenger ribonucleic acid. Mol Endocrinol 2:496–501 (1988).
16. Scroccci LA, Jones LA. Alteration of proto-oncogene c-fos expression in neonatal estrogenized BALB/c female mice and murine cervicovaginal tumor Lj6195. Endocrinology 129:2251–2255 (1991).
17. Nephew KP, Tang M, Khan SA. Estrogen differentially affects c-jun expression in uterine tissue compartments. Endocrinology 134:1827–1834 (1994).
18. Bigsby RM, U A. Differentially regulated immediate early genes in the rat uterus. Endocrinology 134:1820–1826 (1994).
19. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochim Biophys Acta 1072:129–157 (1991).
20. Kovaly K, Bravo R. The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol Cell Biol 11:4406–4417 (1991).
21. NIH. Guide for the Care and Use of Laboratory Animals. NIH Publication No 86–23. Bethesda, MD: National Institutes of Health, 1985.
22. Bigsby RM, Young PCM. Estrogenic effects of the anti-oestrogen on agonist oestradiol (ZK98.293) in the rodent uterus. Am J Obstet Gynecol 171:188–194 (1994).
23. Nephew KP, Peters GA, Khan SA. Cellular localization of estrogen-induced c-fos messenger ribonucleic acid in the rat uterus: c-fos expression and uterine cell proliferation do not correlate strictly. Endocrinology 138:3007–3015 (1995).
24. Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Sisson S, McDonnell DP, Saavo KW. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142:203–214 (1998).
25. Dodge JA, Glasebrook AL, Magee DE, Phillips DL, Sato M, Shott LL, Bryant HU. Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat. J Steroid Biochem Mol Biol 59:155–161 (1996).
26. Gland A, Leroy F, Christian J. Effect of estradiol on cell proliferation and histology of the uterine horns in the estrogen and vagine of mice. J Endocrinol 49:243–252 (1971).
27. Kimura J, Obata T, Okada H. Kinetic analysis of hormone-induced mitosis on epithelial cells of mouse uterus and vagina. Endocrinology 145:291–297 (1994).
28. Grese TA, Dodge JA. Selective estrogen receptor modulators (SERMs). Curr Pharm Des 4:71–92 (1998).
29. Knake JB, Sullivan AJ. Metabolism of bisphenol A in the rat. Toxicol Appl Pharmacol 81:175–184 (1986).
30. Persico E, Scalonia M, Ciociello L, Sica V, Bresciani F, Weisz F. Activation of immediate-early genes by estrogen is not sufficient to achieve stimulation of DNA synthesis in rat uterus. Biochim Biophys Acta 171:287–292 (1990).
31. Nephew KP, Peters GA, Khan SA. Cellular localization of estradiol-induced c-fos messenger ribonucleic acid in the rat uterus: c-fos expression and uterine cell proliferation do not correlate strictly. Endocrinology 136:3007–3015 (1995).  
32. Nephew KP, Bigsby RM. Unpublished data.  
33. Lanahan A, Williams JB, Sanders LK, Nathans D. Growth factor-induced delayed early response genes. Mol Cell Biol 12:3219–3229 (1992).  
34. Stack G, Gorski J. Relationship of estrogen receptors and protein synthesis to the mitogenic effect of estrogens. Endocrinology 117:2024–2032 (1985).  
35. Cheng SW, Pollard JW. c-rasH and ornithine decarboxylase are induced by oestradiol-17β in the mouse uterine luminal epithelium independently of the proliferative status of the cell. FEBS Lett 196:309–314 (1986).  
36. Dean DM, Sanders MM. Ten years after: reclassification of steroid-responsive genes. Mol Endocrinol 10:1489–1495 (1996).  
37. Spearow JL, Doemeny P, Sara R, Leffler R, Barkley M. Genetic variation in susceptibility to endocrine disruption by estrogen in mice. Science 285:1259–1261 (1999).  
38. Lee AE. Cell division and DNA synthesis in the mouse uterus during continuous oestrogen treatment. J Endocrinol 55:507–513 (1972).  
39. Martin L. Estrogens, anti-estrogens and the regulation of cell proliferation in the female reproductive tract in vivo. In: Estrogens in the Environment (McLachlan JA, ed). New York:Elsevier, 1980:103–129.  
40. Roper RJ, Griffith JS, Lytton CR, Doerge RW, McNabb AW, Broadbent RE, Teuscher C. Interacting quantitative trait loci control phenotypic variation in murine estrogen-regulated responses. Endocrinology 140:556–561 (1999).  
41. Gorski J, Wendell D, Gregg D, Chun TY. Estrogens and the genetic control of tumor growth. Prog Clin Biol Res 396:233–243 (1997).