Effects of the Xenoestrogen Bisphenol A on Expression of Vascular Endothelial Growth Factor (VEGF) in the Rat

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Bisphenol-A (BPA) is used to produce polymers for production of polycarbonate and epoxy resins that are used in food containers and dental appliances. BPA binds to estrogen receptors and induces estrogenic activity in a number of biological systems. We recently reported that although Fisher 344 (F344) and Sprague-Dawley (S-D) rat strains exhibit different sensitivities to BPA at the level of vaginal epithelial cell proliferation, there was no difference in immediate early proto-oncogene expression between the two animal strains. In the present study we investigated the effects of BPA on expression of another estrogen-target gene, vascular endothelial growth factor (VEGF), in the uterus, vagina, and pituitary of F344 and S-D rats. Adult rats were ovariectomized and treated with BPA by intraperitoneal injection at concentrations of 0.02 to 150 mg/kg body wt. Expression of VEGF was monitored by RNase protection assay at 2 hr after treatment. There was a significant effect of dose of BPA on the type of VEGF isoform expressed in the uterus, vagina, and pituitary. BPA induced greater ($P < 0.01$) levels of $VEGF_{164}$ and $VEGF_{120+188}$ than $VEGF_{110}$ levels. The lowest BPA dose that had a significant ($P < 0.05$) effect on VEGF expression compared with vehicle treatment was 37.5 mg/kg body wt.; dose-response curves did not differ between strains. This is the first report that the primary response of the uterus, vagina, and pituitary to BPA includes rapid induction of VEGF expression. Due to the capacity of VEGF to engage pleiotropic signaling pathways in other cellular systems, we suggest that modulation of VEFG may play a role in establishing the response of estrogen-target organs to estrogenic xenobiotics.

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Key words: vascular endothelial growth factor; Bisphenol A; xenoestrogen; uterus; vagina; pituitary; rat

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A number of manufactured compounds have been shown to have estrogenic activity (1). Bisphenol-A (BPA; 4,4'isopropylidenediphenol) is one such compound that is particularly abundant in the environment. BPA is a monomer used in the manufacture of polycarbonate plastics, and it is estimated that producers of plastic products in the United States alone have the capacity to manufacture over 2 billion pounds of this compound annually (2). BPA has been shown to bind to estrogen receptors and to induce estrogenic activity in a number of assays, including stimulation of cell proliferation and induction of progesterone receptor expression in breast cancer cells in culture (3, 4), stimulation of proliferation and proto-oncogene expression in rat vaginal epithelium (5, 6), and stimulation of prolactin release from rat pituitary (5, 7). Because BPA can leach from polycarbonate plastics widely used in food packaging and from resins used in dental appliances, there is a general concern for its potential to cause adverse effects on human health (8–10).

Responses of the reproductive tract of ovariectomized rodents, which include changes in gene expression, cellular hypertrophy and DNA synthesis, and vascular changes, have been used extensively to evaluate test compounds for estrogenic activity. Regulation of vascular permeability and blood vessel growth in the mammalian female reproductive tract are associated with changes in gene expression of several angiogenic factors, including vascular endothelial growth factor (VEGF) (reviewed in Refs. 11–13). A single gene encodes VEGF, but alternative exon splicing generates several VEGF isoforms. Splice variants of 110, 115, 121, 145, 165, 189, and 206 amino acids have been reported (14, 15), and the VEGF isoforms are thought to differ in biological function (16, 17). VEGF expression is induced by estrogen in the uterus of many species, including humans (18–21). Furthermore, VEGF is a primary response gene in the rat uterus (22), and peak induction of VEGF expression in the rat uterus occurs within 1 to 3 hr after treatment with female sex steroid hormones (12, 22, 23). Differences in

expression of VEGF isoforms have been reported after 17β-estradiol (estradiol) administration to ovariectomized rats (22–24) and sheep (25). So far, VEGF expression in the mammalian reproductive tract after xenoestrogen (BPA) treatment has not been described.

Several investigators have shown that the response of a target tissue to estrogenic stimuli can be influenced by rodent strain differences (26–31). We recently reported that the vaginal epithelium of the Fisher 344 (F344) inbred strain of rat is more sensitive to BPA stimulation than the out-bred Sprague-Dawley (S-D) strain (6). The effects of genetic background could be reflected by expression profiles of estrogen-target genes such as VEGF, but few studies have examined strain differences in gene expression after xenoestrogen administration. Thus, we carried out the present study to investigate the effect of BPA on expression and modulation of VEGF mRNA in the vagina and uterus of F344 and S-D rats.

Materials and Methods

Animals, Treatments, and Sample Collection. All animal studies were performed under protocols and procedures approved by local Institutional Animal Care and Use Committee, in accordance with NIH standards established by the Guidelines for the Care and Use of Experimental Animals and the American Veterinary Medical Association. Mature (6–8-week-old) female S-D and F344 rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were ovariectomized (OVX) 1 week after arrival and were used in experiments 3 weeks after the surgery. Rats were given animal chow and water *ad libitum* and were maintained on a 12-hr light/dark cycle with lights on at 0600 hr. BPA (Aldrich Chemical Co., Milwaukee, WI) was dissolved in ethanol and diluted in sesame oil. To determine the time course of expression of VEGF mRNAs in response to BPA, a pilot study was carried out in S-D rats ($n = 15$) administered 200 mg/kg body wt. BPA intraperitoneally. Uteri, vaginas, and pituitaries were removed at 0, 2, 3, 6, and 24 hr after BPA treatment, were immediately placed in liquid nitrogen, and were stored at –80°C for ribonuclease protection assays (RPA; described below). Quantitation of the RPA revealed an overall maximal increase in VEGF mRNA level at 2 hr after BPA administration (data not shown). VEGF mRNAs declined slowly thereafter and at 24 hr were similar to control levels. Results of our time course experiment agree with other studies examining the time course of VEGF mRNA expression in the uterus in response to estradiol (22–24). On the basis of this study, animals ($n = 4$ per group) were injected intraperitoneally with various doses of BPA (0.02, 0.2, 2.0, 10, 18.75, 37.5, 75, or 150 mg/kg body wt) or sesame oil vehicle (Sigma, St. Louis) and sacrificed 0 or 2 hr later.

RPA for VEGF. Total RNA was isolated using TRI-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol and as we have described previously (6, 32). Each RNA preparation was assessed visually for integrity by staining with ethidium bromide and electrophoresis in a denaturing 1% agarose gel (6, 32). Only intact RNA was used for the RPA. Concentrations of the final preparations were calculated from A260 reading using a Shimadzu UV-1201 spectrophotometer (6, 32). Reproductive tract tissues were analyzed individually for each animal; however, pituitaries were pooled by treatment group prior to analysis.

The 570-bp rat VEGF164 cDNA in pBluescript was provided Dr. S. Hyder (University of Texas Medical Center, Houston, TX). The plasmid was linearized using BamHI. Antisense riboprobes were generated from linearized templates using the MAXIscript Kit (Ambion, Austin, TX), T7 RNA polymerase, and the incorporation of α-^{32}P-UTP (New England Nuclear, Boston, MA; 800 Ci/mmol) according to the manufacturer's protocol. The VEGF template generated a 590-bp probe, containing 570 bp of antisense VEGF and 20 bp of vector sequence. An antisense riboprobe specific for rat cyclophilin was used to normalize lanes for differences in loading among lanes. This probe was generated from the template pTRI-cyclophilin (Ambion; a probe length of 165 nucleotides) and produced a protected fragment of 103 nucleotides. Cyclophilin mRNA expression does not appear to be upregulated by estrogens in the rat uterus (33, 34). An RNA molecular weight marker (Century Marker Template; Ambion) was also run in each gel, using T7 RNA polymerase to produce marker transcripts of lengths of 100, 200, 300, 400, and 500 bp.

VEGF mRNA was quantified in uterine, vaginal, and pituitary samples using the RPA II kit (Ambion), as we have previously described in detail (6, 32). After hybridization and RNase digestion, the protected hybrids were separated on 6% polyacrylamide denaturing gel. The gel was dried and exposed to x-ray film (Biomax AR, Eastman Kodak Company, Rochester, NY). The optical densities (OD) of autoradiograms from the protected bands of the RNase protection assay for VEGF isoforms and cyclophilin of the individual animals were measured and quantified using an imaging densitometer and Molecular Analyst Software (GS670, Bio-Rad, Hercules, CA). The mRNA levels for VEGF were normalized against cyclophilin mRNA levels by dividing the OD of the autoradiographic band by the OD of the corresponding cyclophilin band of each specimen, and the results were expressed as arbitrary units.

Statistical Analysis. A general linear model procedure with repeated measures was used to examine the relationship among VEGF expression and strain, type of VEGF isoform, and dose level. Analyses were performed separately on data collected from the uterus and vagina. The ANOVA was followed by Dunnett's t test to identify the dose at which VEGF mRNA levels differed from the control mean. Statistical significance is noted where $P < 0.05$.

Results and Discussion

The known VEGF gene splice variants and the regions of their transcripts that would be protected by the RNA

probe used in this study are described in Fig. 1. The mammalian VEGF gene consists of at least eight exons, and alternative splicing results in a number or variant mRNA molecules that have the potential to encode different proteins with biological activity (14–17, 35).

The predominant forms of VEGF in tissues from adult rats include transcripts encoding $VEGF_{120}$,$VEGF_{164}$, or $VEGF_{188}$ amino acid-containing proteins (22, 36). $VEGF_{144}$ and $VEGF_{110}$ splice variants in tissues have been described, but are of much lower abundance (16, 37, 38). In human tissues, virtually identical VEGF splice variants have been described, but generally contain one more amino acid than the rodent counterpart (12, 13, 39, 40). In addition, a $VEGF_{206}$ has been described in human fetal liver and placenta (37, 38).

We used RNase protection assay to determine the level of VEGF mRNAs splice variants expressed in uterus, vagina, and pituitary after BPA treatment of F344 and S-D rats. We observed protected RNA fragments of 570, 419, 312, and 260 nucleotides in length (Fig. 2). The undigested probe ran as a 590-bp fragment in separate experiments, and in preliminary tests the probe produced no bands after hybridization with tRNA and subsequent digestion with RNase (data not shown). On the basis of sequence comparison of the probe we used for RPA to the previously described splice variants of VEGF mRNA (14–16, 18, 35), we concluded that the protected fragments represented $VEGF_{164}$, $VEGF_{120+188}$, and $VEGF_{110}$ (see Fig. 1). Our results using RPA agree with those studies and others (22, 24) using reverse transcription-PCR to examine VEGF expression in the rat uterus. Because the RPA probe used in the present study protected equally sized nucleotide bands for both the 188- and 120-amino acid forms of VEGF, we were unable to distinguish between expression patterns of $VEGF_{188}$ and $VEGF_{120}$; however, the signal was most likely due to $VEGF_{120}$ expression, based on reports that $VEGF_{120}$ is more highly abundant than $VEGF_{188}$ in rat reproductive tissues, including uterus and pituitary (22, 24, 36). $VEGF_{110}$ has been described in rat tissues and has the unusual characteristic of retaining the first three exons, instead of the first five, as well as having an apparent splice within the seventh exon (36). The small, approximately 260-bp band detected by our probe is either nonspecific or another yet to be characterized isoform. A $VEGF_{144}$ splice variant of low abundance in tissues of rats and other species has been described (18, 22, 36–38), but was not detected by the probe used in this study.

There was a significant interaction effect between dose of BPA and type of VEGF isoform for both the uterus ($P < 0.01$) and vagina ($P < 0.01$; Fig. 3). $VEGF_{164}$ and $VEGF_{120+188}$ were more abundant than $VEGF_{110}$ in both the uterus and vagina, and our finding agrees with the observations of others in rat reproductive tissues (22, 24, 36). The function served by either VEGF isoform in the uterus and

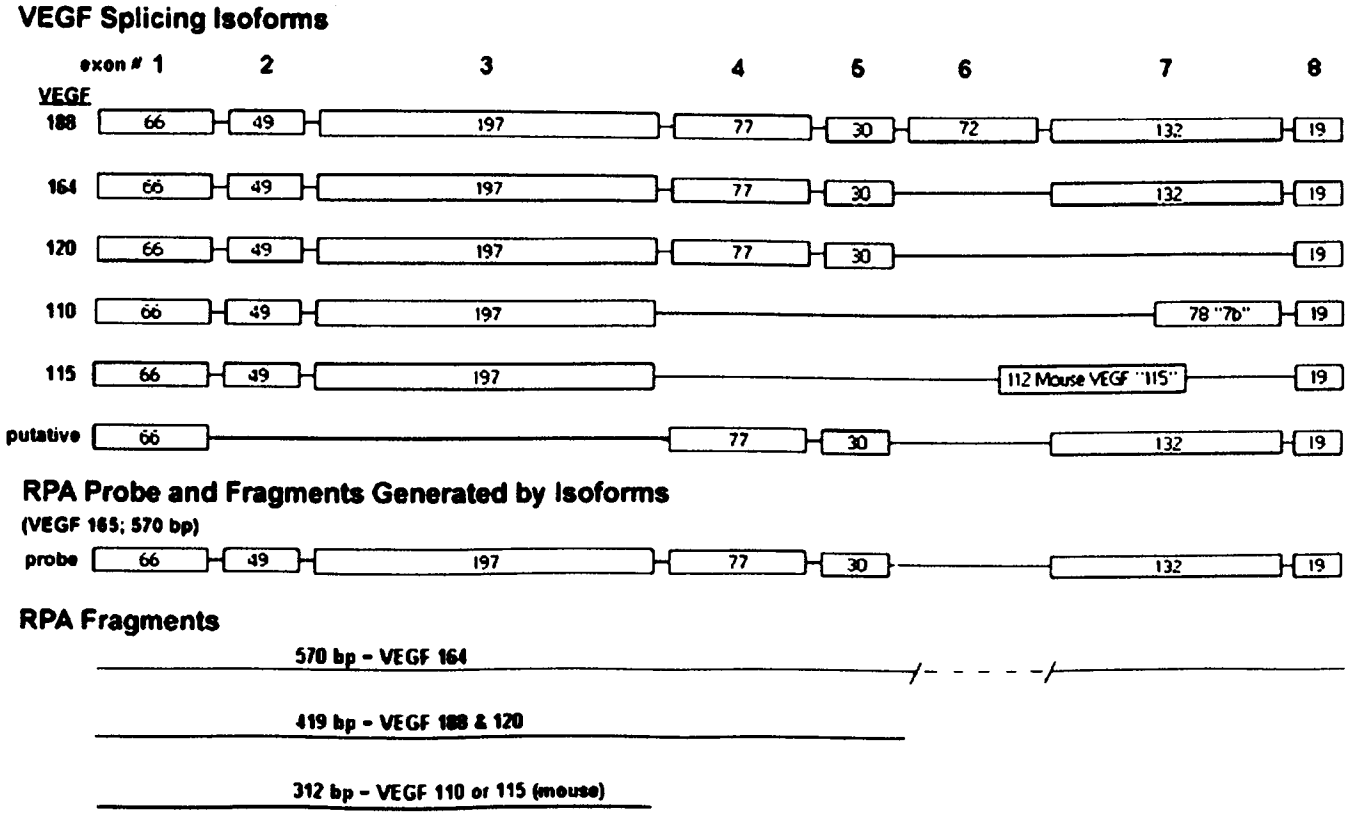

Figure 1. Schematic model for the generation of molecular species of VEGF by alternative splicing of mRNA is shown in the top panel. The probe used for RNase protection assays in the present study is shown in the middle panel, and the corresponding protected fragments are shown in the bottom panel.

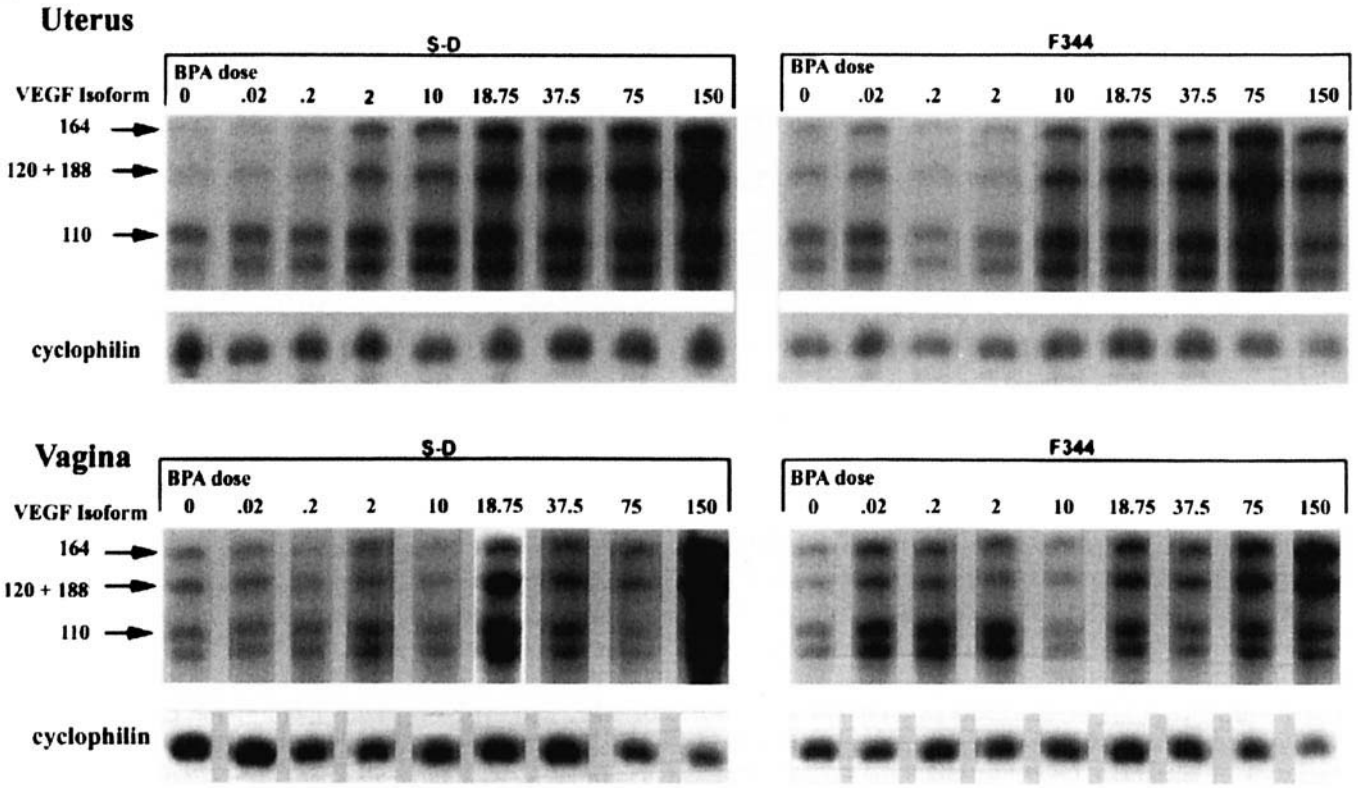

Figure 2. Dose response for BPA induction of VEGF isoforms in S-D and F344 rat uterus (top) and vagina (bottom). Mature, ovariectomized rats (n = 4 per dose) were injected intraperitoneally with various doses of BPA (0.02, 0.2, 2.0, 10, 18.75, 37.5, 75, or 150 mg/kg body wt) or sesame oil. Total RNA was extracted at 2 hr after BPA administration and subjected to RNase protection assays. Representative autoradiograms for VEGF and cyclophilin are shown. Lanes represent individual samples obtained at the indicated doses of BPA treatment.

vagina, however, remains unknown. VEGF likely plays a role in the striking alterations in vascular permeability and changes in neovascularization observed in the uterus during the reproductive cycle (41). Both $VEGF_{120}$ and $VEGF_{164}$ can induce endothelial cell proliferation and increase permeability, although differences in their heparin-binding properties, potencies, and tissue distribution have been reported (16, 17). Although $VEGF_{110}$ is seemingly rare and of low abundance in rat tissues, it has a potential to produce a functional VEGF peptide, based on the observation that this type of variant has also been reported as a functional peptide, $VEGF_{115}$, in the mouse (40).

VEGF expression is more sensitive to BPA in the uterus than in the vagina (Fig. 3). In the uterus, when compared with control levels, the lowest dose of BPA that had a significant effect ($P < 0.05$) on VEGF expression was 37.5 mg/kg. In the vagina, however, it was not until the dose of 150 mg/kg BPA that $VEGF_{164}$ and $VEGF_{120+188}$ levels differed significantly ($P < 0.05$) from control. Levels $VEGF_{110}$ in the vagina were never significantly different than control at any dose used in this study.

We did not observe strain differences in sensitivity to BPA-induced VEGF expression; the patterns of the dose-response curves were similar for both strains of rat. In addition, the time course and dose response for BPA-induced VEGF expression in these strains of rats is essentially similar to what we observed for c-*fos* (6). Thus, it seems unlikely that VEGF plays a role in the different sensitivities of the uterus or vagina to estrogenic stimuli in these two strains of rats. However, the fact that vaginal response required a substantially larger dose of BPA reinforces the concept that tissue differences must be taken into account when utilizing rat reproductive tract tissues to assess the estrogenic activity of a test compound.

The pituitary gland is an established target of estrogens, and it was of interest to determine if BPA could induce VEGF expression in the pituitary. As seen in Fig. 4, BPA induced VEGF mRNA expression in the pituitary in a dose-responsive manner. Higher expression levels of $VEGF_{164}$ and $VEGF_{120+188}$ compared with $VEGF_{110}$ were seen after treatment with 37.5, 75, and 150 mg/kg body wt BPA. The pituitary response to BPA was similar in both F344 (Fig. 4) and S-D rats (data not shown). In addition, induction of VEGF in the pituitary was rapid, within 2 hr after a single injection of BPA (data not shown), and similar to the dynamics we observed in the vagina and uterus and to what was observed for estradiol-induced VEGF expression in the rat pituitary (24). Interestingly, VEGF expression in the pituitary was upregulated by estradiol only in the anterior lobe (24). Although the function of VEGF in the pituitary is not known, the gland is a highly vascularized structure with a unique hypothalamic-pituitary portal (HPP) system that plays a key role in its physiology. VEGF may play a role in maintenance and regulation of this vascular network or act

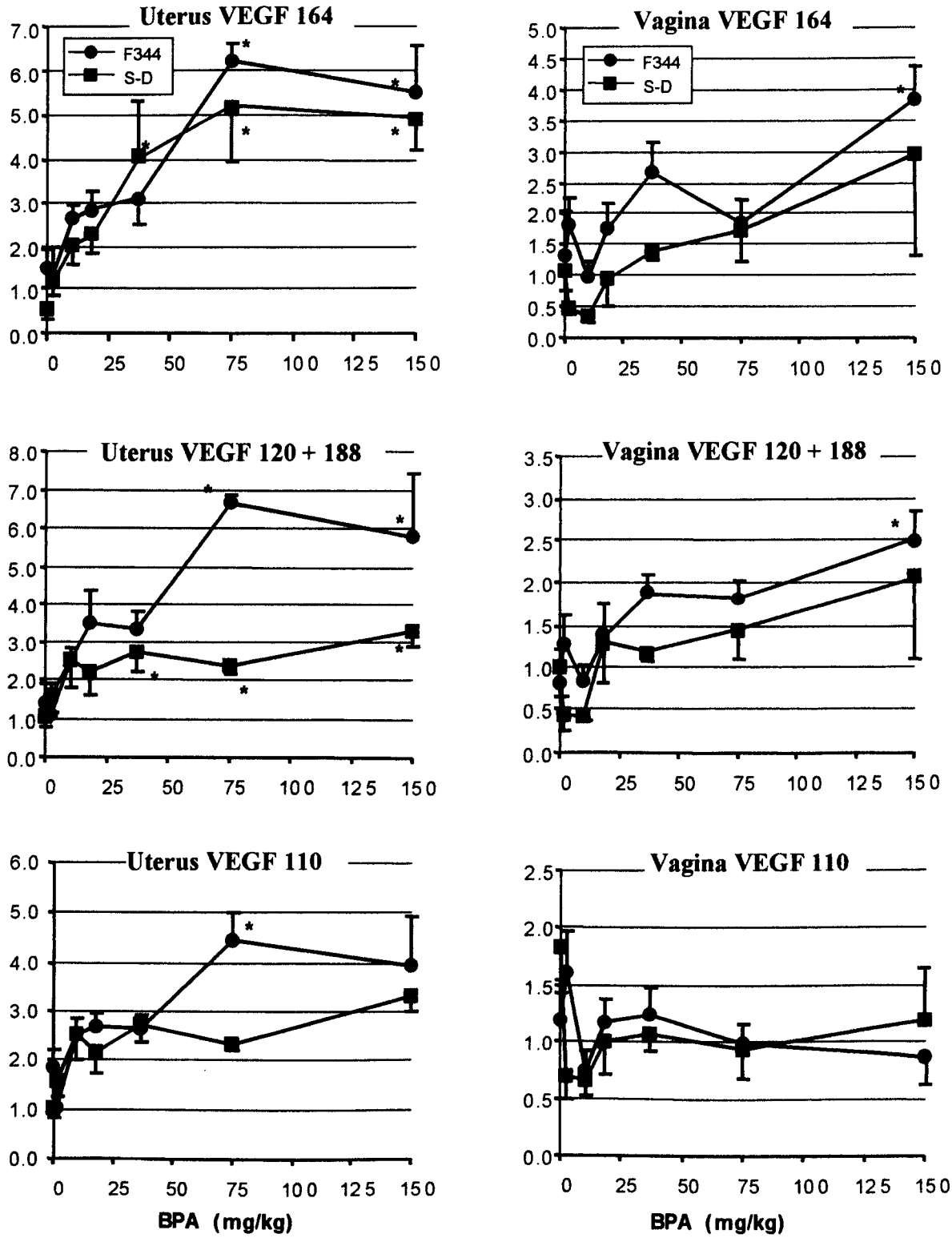

Figure 3. Effect of BPA dose on VEGF isoform expression. VEGF mRNA levels for uterus (left) and vagina (right) are depicted graphically on the y axis as arbitrary OD units relative to cyclophilin mRNA. VEGF expression was analyzed separately in F344 (●) and Sprague-Dawley (■) rats. Values are means ± SEM, $n = 3$; asterisks indicate $P < 0.05$ versus the corresponding control (0 dose).

as a mediator of vascular permeability of the HPP system and thereby play a role in the exchange of hormones between the blood and the pituitary.

Our studies are the first demonstration that BPA will rapidly stimulate expression of VEGF mRNA splice variants in classic estrogen-responsive tissues of the rat. It has been well established that BPA produces estrogenic responses in uterotrophic assays (42), and our observations

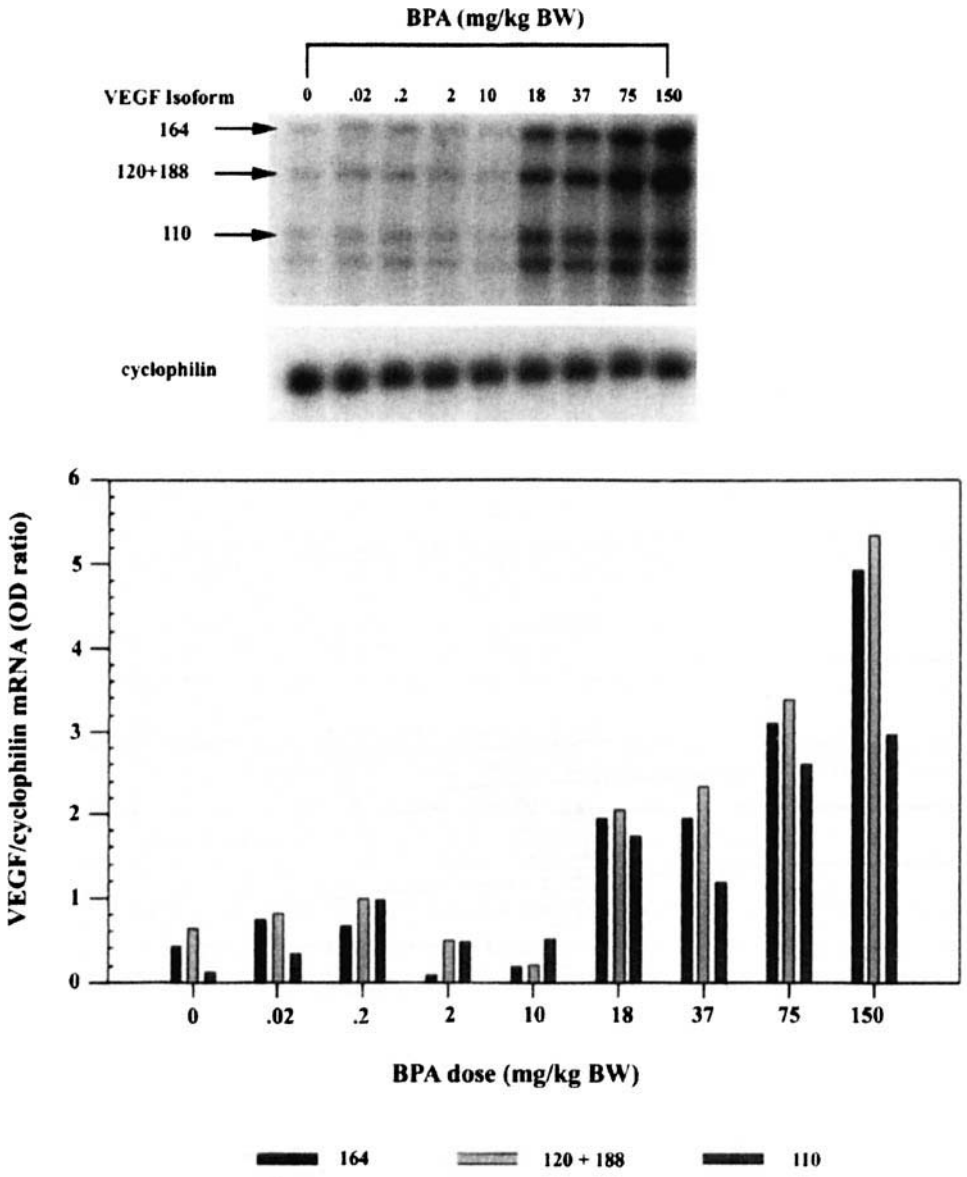

Figure 4. BPA induction of VEGF isoform expression in the pituitary. Two hours after BPA treatment, pituitaries were collected and pooled for three animals in the same dose group. Total RNA was extracted from the pooled pituitary samples and subjected to RPA for both VEGF and cyclophilin. The RPA autoradiogram from F344 rat pituitaries is shown on the upper half. The dose-response effect was analyzed by densitometric analysis of the autoradiogram and depicted graphically on the lower half.

suggest that BPA may act as an endocrine disruptor directly in several target organs, e.g., uterus, vagina, and pituitary, as well as indirectly by altering pituitary function and perhaps hypothalamic-pituitary interactions. This study and others may have far reaching implications, given the high abundance of BPA in the environment, the potential for exposure to BPA, the unique ability of VEGF to promote so many events necessary for angiogenesis, and observations that VEGF activity is associated with the progression of important pathologies, including cancer (17).

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