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Bisphenol A Induces Both Transient and Permanent Histofunctional Alterations of the Hypothalamic-Pituitary-Gonadal Axis in Prenatally Exposed Male Rats

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Exposure to bisphenol A (BPA) *in utero* has been shown to induce alterations in the prostate of 30-d-old Wistar rats. Herein, we examine both the time course of BPA action on the rat prostate and the effects of BPA on the male hypothalamic-pituitary-gonadal axis. This was achieved by exposing rats to BPA *in utero*, followed by immunohistochemistry and morphometric analysis of prostatic tissue, evaluation of estrogen receptor-α (ERα) and ERβ mRNA expression in both the preoptic area (POA) and medial basal hypothalamus, and determination of PRL, LH, and testosterone serum levels. On d 30 (peripubertal period), the prostatic periductal stroma of BPA-exposed rats demonstrated a significantly larger layer of fibroblasts than that of controls, whereas on d 120 (adulthood) no significant differences were observed. Moreover, BPA-exposed rats on d 15 exhibited an increase in stromal cellular proliferation compared with controls. Decreased expression of both androgen receptor in prostatic stromal cells and prostatic acid phosphatase in epithelial cells was observed only on d 30 in BPA-exposed males. BPA did not alter POA ERα mRNA expression, whereas a 4-fold increase in POA ERβ mRNA expression was observed on both d 30 and 120. No alterations were observed in either ERα or ERβ expression in the medial basal hypothalamus. BPA-exposed males exhibited increased PRL levels only on d 30, whereas a transient increase in serum testosterone levels was observed on d 15. These results support the hypothesis that prenatal exposure to environmental doses of BPA induces both transient and permanent age-dependent alterations in the male reproductive axis at different levels. (*Endocrinology* 144: 3206–3215, 2003)

XENOESTROGENS HAVE BEEN suggested to disrupt the internal signaling network that regulates reproductive development and function in animals and humans (1). In males, environmental exposure to estrogenic compounds has been associated with a high incidence of reproductive disorders such as cryptorchidism, decreased sperm count, and erectile dysfunction (2, 3). Bisphenol A (BPA) is a xenoestrogen used in the manufacture of polycarbonate plastics and epoxy resins from which a variety of products are made, including reusable milk and food storage containers, baby formula bottles, the interior lacquer-coating of food cans, and dental sealants and composites. Studies have shown that incomplete polymerization of these products during manufacture and/or depolymerization due to increased temperatures (induced either intentionally for sterilization purposes or unintentionally during storage in warehouses) causes BPA and its derivatives to leach into foods (4–23 μg/can), beverages (7–58 μg/g), and saliva (90–913 μg/saliva collected in a 1-h period after application of dental sealant) in concentrations that are sufficient to induce the proliferation of estrogen target cells in culture (4–6). These data indicate that humans are exposed to BPA. Recently, concentrations of BPA during pregnancy have been reported in serum (from 0.3–18.9 ng/ml), follicular fluid (1–2 ng/ml), fetal serum (0.2–9.2 ng/ml), amniotic fluid (8.3 ± 8.7 ng/ml), and placental tissue (1.0–104.9 ng/g) (7, 8). These reports showed that BPA is present in serum during pregnancy as well as in fetal serum and full-term amniotic fluid, confirming passage through the placenta.

In laboratory animals, prenatal exposure to BPA and arochlor was shown to modify anogenital distance (AGD), increase prostate size, decrease epididymal weight, and alter estrous cyclicity and plasma LH levels (9–11). Using low doses of BPA, Steinmetz *et al.* (12) induced hyperprolactinemia in ovariectomized Fisher 344 rats. In the same rat strain, Khurana *et al.* (13) showed that exposure of newborns to either BPA or octylphenol induced both delayed and prolonged hyperprolactinemia. Moreover, they reported alterations in estrogen receptor-α (ERα) mRNA expression in the medial basal hypothalamus (MBH) of female neonates, whereas no differences in hypothalamic ERα mRNA expression were observed in male pups. BPA-induced hyperprolactinemia has been postulated to be an indirect mechanism that could increase the incidence of prostate inflammation in the adult rat (14).

In a previous report (15) we demonstrated that *in utero* exposure to environmentally relevant levels of BPA decreased androgen receptor (AR) expression and modified prostate periductal stromal cell phenotype at d 30 in Wistar rats. In addition, we observed a decrease in prostatic acid phosphatase (PAP) expression, suggesting alterations in prostatic functional activity. Numerous efforts have focused on elucidating the effects of estrogenization during early

Abbreviations: AGD, Anogenital distance; AR, androgen receptor; BPA, bisphenol A; BrdU, bromodeoxyuridine; DMSO, dimethylsulfoxide; ER, estrogen receptor; LHRH, LH-releasing hormone; MBH, medial basal hypothalamus; PAP, prostatic acid phosphatase; POA, preoptic area; α-SMA, α-smooth muscle actin; T, testosterone; Vim, vimentin.

postnatal life on prostate development and function (16, 17). These studies demonstrated that several effects of neonatal estrogenization on male puberty and the male reproductive tract are transient and that the reversibility of the process is a function of the dose, the end points examined, and the rat strain employed (18, 19). However, little is known about the time course of the alterations observed in males prenatally exposed to environmentally relevant doses of xenoestrogens. Taking into account the above-mentioned findings, the first goal of the present study was to assess the time course of histofunctional disruption of the rat ventral prostate induced by prenatal BPA exposure. The second goal was to evaluate the action of BPA on the male hypothalamic-pituitary-gonadal axis by evaluating several different end points. ERα and ERβ mRNA expression in the preoptic area (POA) and MBH were scanned using an RT-PCR technique. PRL, LH, and testosterone (T) serum levels were also determined to ascertain whether these parameters were differentially affected.

Materials and Methods

Animals and experimental design

Sexually mature female rats of an inbred Wistar-derived strain were bred at the Department of Human Physiology (Santa Fe, Argentina). Animals were maintained under a controlled environment (22 ± 2 C; lights on from 0600–2000 h) and had free access to pellet laboratory chow (Constantino, Córdoba, Argentina) and tap water supplied from glass bottles. All rats were handled in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences.

Proestrous females were caged overnight with males of proven fertility. Day 1 of pregnancy was designated as the day that sperm were found in the vagina. Pregnant rats were placed into three experimental groups: dimethylsulfoxide (DMSO) vehicle-treated (control), 25 μg/kg body weight/d BPA (25-BPA), and 250 μg/kg body weight/d BPA (250-BPA).

Timed pregnant rats were assigned to each group (n = 7–9 mothers/treatment group) and then individually housed in stainless steel cages. In our colony, delivery occurs on d 23 between 1230–1400 h. On gestation d 8, a miniature osmotic pump (model 1002, Alza Corp., Palo Alto, CA) was inserted sc over the spine caudal into the scapula. Osmotic pumps were filled with either 25-BPA or 250-BPA (Sigma-Aldrich Corp., St. Louis, MO) dissolved in DMSO (99.9%, molecular biology grade, Sigma-Aldrich Corp.) or only with DMSO in control rats. BPA or its vehicle was administered continuously from d 8 of gestation to the day of parturition (d 23). The BPA solutions were released at a rate of 0.25 μl/h. No signs of acute or chronic toxicity were observed, and no significant differences in weight gain between BPA-exposed and control mothers were recorded during gestation. No differences in litter size or pup body weight were observed at birth or at weaning. Moreover, sex ratios of the litters were comparable in the three groups, and AGD measured at birth and on postnatal d 4 did not differ among females or males of different groups (data not shown).

After parturition, pups were weighed and sexed according to AGD, and litters of eight pups (preferably four males and four females) were left with lactating mothers until sacrifice or weaning on postnatal d 22. Males from a single mother were killed on selected postnatal days representative of prepuberty (d 15), peripuberty (d 30), and adulthood (d 120). There were six to eight animals per group at each time point evaluated. Siblings were excluded from the same experimental group. Pups were injected with bromodeoxyuridine (BrdU; Sigma-Aldrich Corp.; 6 mg/100 g body weight/1.5 ml PBS, ip) 2 h before sacrifice. Animals were killed by decapitation, trunk blood was collected, and serum stored at −20 C until used for hormone assays.

Ventral prostates were microdissected, weighed, and fixed by immersion in 10% formalin buffer for 6 h at room temperature. Fixed tissue was dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Serial sections (5 μm thick) of ventral prostate were mounted on 3-aminopropyl triethoxysilane (Sigma-Aldrich Corp.)-coated slides and dried for 24 h at 37 C. For each prostate specimen, three sections separated at 20-μm intervals were evaluated. To secure uniformity between sections of each animal, a nonparametric ANOVA between sections of the same specimen was performed.

From d 30 and 120 rats, brain tissue blocks containing mainly the POA or MBH were quickly microdissected under a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY). The POA fragment was encompassed by the anterior portion of the anterior commissure, the beginning of the ascending optical tracts, and laterally by a virtual line that is projected from the internal capsule to the external edge of the optical tracts. The MBH fragment was delimited by the beginning of the ascending optical tracts and the mammillary bodies. All microdissections were performed using thick coronal sections so that the ventral portion of the third ventricle was always visible (20). After removal, tissue samples were immediately frozen in liquid nitrogen and stored at −80 C until used for RNA analysis.

Immunohistochemistry and morphometric analysis

The expression of several markers was evaluated by immunohistochemistry to characterize the cellular phenotype and biological behavior of the prostatic tissue (15, 21). Incubation with primary antibodies was performed at 4 C for 14–16 h (Table 1). Antigens were stained using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp.), and sections were counterstained with Mayer's hematoxylin (Biopur, Rosario, Argentina). Each immunohistochemical run included both positive and negative controls. Negative controls were incubated with nonimmune mouse or rabbit serum (Sigma-Aldrich Corp.).

BrdU incorporation into proliferating cells was determined by immunohistochemistry (22) and evaluated in both epithelial (basal and glandular) and stromal cell nuclei. Zonation of the prostate tissue was performed as previously described (15). The periductal stromal zone of each duct was defined as a circular area 18 μm wide around the duct (projecting from the basement membrane toward the outer layers). The total periductal area was calculated using digital image analysis software (Image Pro-Plus 4.1.0.1 system, Media Cybernetics, Silver Spring, MD) for 60 ducts in each histological section using a Dplan ×40 objective lens with a reticule in the eyepiece. All immunostained epithelial and stromal nuclei within the defined regions, regardless of intensity, were scored as positive. Positive cells were expressed as the percent ratio of the total number of epithelial or stromal cells evaluated in the ventral prostate.

Image analysis of immunostained tissue sections was performed by color segmentation analysis as previously described (15). Briefly, cy-

TABLE 1. Antibodies used for immunohistochemistry[a]

Antibody	Animal source	Clone	Company	Dilution
BrdU	Mouse	85-2C8	Novocastra (Newcastle upon Tyne, UK)	1:400
Vim	Mouse	V9	Novocastra (UK)	1:100
α-SMA	Mouse	αsm-1	Novocastra (UK)	1:50
ERα	Mouse	6F-11	Novocastra (UK)	1:60
AR	Rabbit		Affinity BioReagents (Golden, CO)	1:120
PAP	Rabbit		Sigma (St. Louis, MO)	1:400

[a] Specificity of antibodies used has been tested by the suppliers and by us using Western blot assays of tissues containing the proteins under investigation.

TABLE 2. Sequence of primers used in RT-PCR experiments

Primer name	Sequence	PCR product size (bp)
ERα sense	5′-AAT TCT GAC AAT CGA CGC CAG-3′	
ERα antisense	5′-GTG CTT CAA CAT TCT CCC TCC TC-3′	345
ERβ sense	5′-TTC CCG GCA GCA CCA GTA ACC-3′	
ERβ antisense	5′-TCC CTC TTT GCG TTT GGA CTA-3′	262
L19 sense	5′-GAA ATC GCC AAT GCC AAC TC-3′	
L19 antisense	5′-ACC TTC AGG TAC AGG CTG TG-3′	290

toskeletal protein expression was quantified in the periductal stroma using an automated standard sequence programmed by Auto-Pro macro language. Using consecutive histological sections, the automatic script was performed to measure the percentage of the reference periductal area (relative area) occupied by vimentin (Vim) or α-smooth muscle actin (α-SMA) cells. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction) and by calibrating the measurement system with a reference slide to determine background threshold values. The reference slides contain a series of tissue sections stained in the absence of primary antibody. The image resolution was set at 640 × 480 pixels, and the final screen resolution was 0.103 μm/pixel.

For ERα- and AR-positive cell characterization, 2 sections were evaluated for each prostate specimen, and 30 representative fields in each section were scored using a Dplan ×40 objective. Positive cells were expressed as the percent ratio of the total number of epithelial or stromal cells measured in the examined area of the ventral prostate.

To obtain quantitative data regarding PAP expression in ductal epithelial cells, 2 sections for each prostate specimen were evaluated, and 30 representative fields in each section were digitalized and recorded using a Dplan ×40 objective. Using Auto-Pro macro language, a second script was created to measure the OD as previously described by Ramos *et al.* (15). All epithelial cells with positive OD values were considered PAP-positive cells. Results were expressed as the percent ratio of the total number of epithelial cells measured in the examined area.

RNA analysis by RT-PCR

An optimized RT-PCR protocol was employed to analyze the relative expression levels of ERα and ERβ mRNAs in the POA and MBH on d 30 and 120 in rats in all experimental groups. Total RNA was isolated using the single step, acid guanidinium thiocyanate-phenol-chloroform extraction method (23). Equal quantities (4 μg) of total RNA were reverse transcribed into cDNA with avian myeloblastosis virus reverse transcriptase (12.5 U; Promega Corp., Madison, WI) using 200 pmol random primers (Promega Corp.). Twenty units of ribonuclease inhibitor (RNAout, Invitrogen, Buenos Aires, Argentina) and 100 nmol of a deoxy NTP mixture were added to each reaction tube in a final volume of 30 μl of 1× avian myeloblastosis virus reverse transcriptase buffer. RT was performed at 42 C for 90 min. Reactions were terminated by heating at 97 C for 5 min and cooling on ice, followed by dilution of the reverse transcribed cDNA with ribonuclease-free water to a final volume of 60 μl. RNA incubated under identical conditions, but without reverse transcriptase, served as a negative control. Primer pairs used for amplification of the target genes are shown in Table 2. The primers were selected based upon a previous reference (24) according to the published cDNA sequences of the rat ERα (25), ERβ (26), and L19 ribosomal protein (27). All amplifications were performed in duplicate. To perform comparative PCR, aliquots of cDNA samples equivalent to 800 ng total RNA input were used in each PCR amplification. Each reaction mixture contained 2.5 U *Taq* DNA polymerase (Promega Corp.), 2.5 mM $MgCl_2$ (Promega Corp.), 0.2 mM of each of the four deoxy-NTPs (Promega Corp.), and 20 pmol of each primer (Invitrogen) in a final volume of 50 μl of 1× PCR *Taq* buffer. After initial denaturation at 97 C for 5 min, the reaction mixture was subjected to successive cycles of denaturation at 96 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min. A final extension cycle at 72 C for 15 min was included. The optimal number of cycles for each reaction was determined experimentally to yield linear relationships between signal intensity and cycle number (Fig. 1). The optimal number of cycles was 36 for ERα and ERβ and 20 for L19 ribosomal protein, using separate reactions for each target gene.

TABLE 3. Mean ± SEM of absolute ventral prostate weights at necropsy

Age	Experimental group		
	Control	25-BPA	250-BPA
d 15	22.46 ± 5.12	23.57 ± 3.53	21.79 ± 4.36
d 30	119.4 ± 7.96	115.46 ± 9.27	113.28 ± 8.59
d 120	474.64 ± 34.4	470.30 ± 36.1	462.45 ± 43.9

In all assays negative controls using RNA without RT and *Taq* polymerase-negative tubes were performed to minimize the introduction of potential artifacts. All PCR products were cloned using the TA cloning kit (Invitrogen), and specificity was confirmed by DNA sequencing (data not shown).

The generated cDNA fragments were resolved on 1.5% agarose gels containing ethidium bromide (Sigma-Aldrich Corp.); molecular weights were determined by comparison with molecular weight standards (Cien Marker, Biodynamics, Buenos Aires, Argentina). Agarose gel images were digitized using a Sony ExwaveHAM color video camera (Sony Electronics, Inc., Park Ridge, NJ) and the Image Pro-Plus 4.1.0.1 image system analyzer (Media Cybernetics, Silver Spring, MD). The absolute OD for each PCR product was obtained by densitometry. Values for ERα and ERβ PCR-amplified sequences were normalized with respect to that of the L19 ribosomal protein, allowing relative levels of the specific mRNAs to be expressed in arbitrary units.

Hormone assays

Serum levels of PRL and LH were determined by RIA using the kit provided by the NIDDK as previously described (28, 29). Results were expressed in terms of rat PRL (RP3) and rat LH (RP3) reference preparations. Intra- and interassay coefficients of variation for PRL were 8.1% and 11.4%, respectively, whereas coefficients for LH were 5.4% and 9.8%, respectively. The lowest detectable levels were 0.039 ng/ml for PRL and 0.016 ng/ml for LH. T concentrations were determined by RIA using [1,2-N-^{3}H]T (60 Ci/mmol; NEN Life Science Products, Boston, MA) and a specific antibody (Immunotech Diagnostic, Montréal, Canada) as previously described and validated by Suescun *et al.* (30). The sensitivity of the assay was 12 pg/ml, and the intraassay coefficients of variation were 7.1%.

Data analysis

Statistical analyses were performed using the Kruskal-Wallis one-way ANOVA, and significance between groups was determined using the Dunn's posttest. $P < 0.05$ was accepted as indicating a significant difference between groups. Nonlinear correlations were determined by sigmoidal approximation (31).

Results

Prenatal BPA exposure induces transient changes in stromal cell phenotype and proliferative activity in the ventral prostate

The absolute weights of the ventral prostates are presented in Table 3. There were no significant differences in the weights of ventral prostates between BPA-treated and control animals at any of the ages studied. At a histological level,

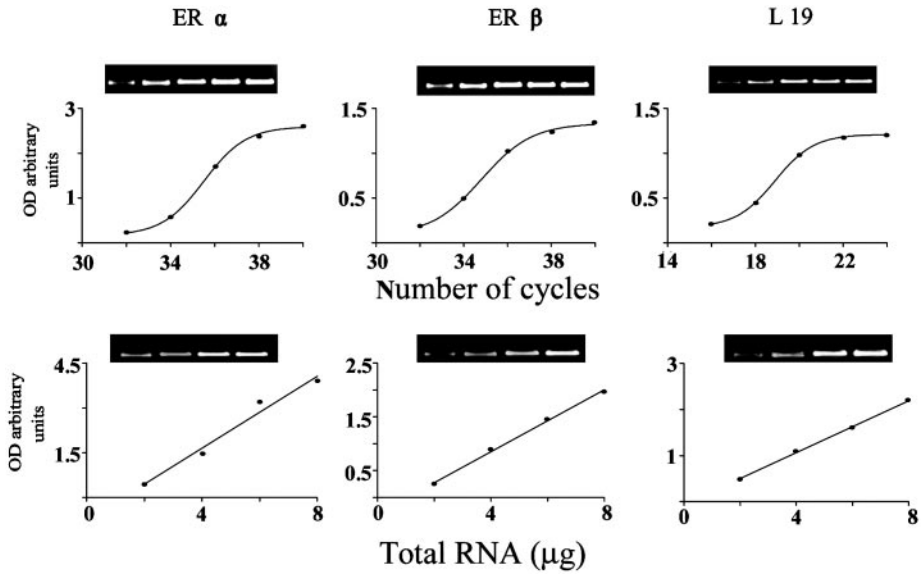

FIG. 1. Optimization of RT-PCR conditions employing a pool of control and treated rat hypothalamic RNA. Quantitative analysis of the cycle dependency (*upper panel*) for the PCR signals revealed a strong linear relationship between cycles 32 and 38 for ERα and ERβ and between cycles 16 and 22 in the case of L19 ribosomal protein ($r^2 > 0.97$). The *lower panel* shows a linear dose dependency of the intensity of PCR signals for each target message. Representative ethidium bromide-stained agarose gels of the DNA products are presented in both panels.

the stroma surrounding the epithelial ducts of the adult control prostate is primarily comprised of α-SMA$^+$ cells that encompass the basal membrane of the epithelial ducts. In the distal tips, the smooth muscle cell layer is separated from the basal membrane in a discontinuous fashion by a sheath of fibroblasts along the prostatic ducts. This cellular arrangement allows the smooth muscle cells to come into direct contact with the epithelium. In the proximal tips, the covering fibroblasts became multilayered such that the smooth muscle cells directly contacted the ductal basal membrane sporadically. The fibroblastic sheath was recognized in serial histological sections by the staining pattern of the Vim$^+$/α-SMA$^-$ cells. On postnatal d 15, no differences were observed between BPA-exposed animals and controls in the relative area occupied by Vim$^+$ (Fig. 2, A *vs.* D) and/or α-SMA$^+$ (Fig. 2, G *vs.* J) cells. In contrast, on d 30 in BPA-exposed groups, the Vim$^+$/α-SMA$^-$ cells (fibroblasts) exhibited a uniform, continuous halo surrounding the proximal and distal ducts; the halo was thicker around the proximal ducts than around the distal ducts (Fig. 2E). In addition, the α-SMA$^+$ cells (smooth muscle cells) formed a discontinuous layer in both distal and proximal ducts; these zones were thinner on d 30 in BPA-exposed animals (Fig. 2K) than in controls (Fig. 2H). In proximal and distal regions on d 30, the relative area occupied by Vim$^+$/α-SMA$^-$ cells in the periductal ring was significantly larger in BPA-exposed groups than in controls (Fig. 3A). On the same day postnatally, the relative area occupied by α-SMA$^+$ cells was smaller in BPA-exposed groups than in controls (Fig. 3B). No differences were observed in the relative areas occupied by Vim$^+$ or α-SMA$^+$ cells between 25-BPA- and 250-BPA-exposed groups. During adulthood (d 120) the periductal stroma was mainly comprised of smooth muscle cells that maintained close contact with the epithelial basal membrane in both distal and proximal tips (Fig. 2, C and I). The enlarged sheath of fibroblasts observed in BPA-exposed males on d 30 was absent on d 120 (Fig. 2F). Furthermore, on d 120 the relative area occupied by Vim$^+$/α-SMA$^-$ or α-SMA$^+$ cells did not differ in either the distal or proximal region between BPA-exposed and control groups (Fig. 3, A and B, and Table 4).

Prenatal BPA exposure did not modify prostate epithelial proliferative activity at any of the ages studied (Fig. 3C). However, a significant increase in cellular proliferation was detected in the periductal stroma of d 15 BPA-exposed rats (Fig. 3D). Proliferative indexes in periductal stroma during the peripubertal period (d 30) and adulthood (d 120) were not affected by BPA exposure (Table 4).

Prenatal BPA exposure induces transient changes in AR and PAP expression in the ventral prostate, whereas ERα was not altered

Epithelial and stromal compartments were negative for ERα in all groups studied (Table 4). Epithelial cells (basal and glandular) were strongly stained for AR in both proximal and distal prostate regions in all groups studied. No significant differences were observed between groups (data not shown). On postnatal d 15, more than 50% of the periductal stroma cells were AR$^+$ in all groups studied (Fig. 3E). The AR staining intensity was higher in smooth muscle cells than in fibroblasts. On d 30 in controls, more than 60% of the periductal stromal cells remained AR$^+$; however, a significant decrease in AR$^+$ stromal cells was observed in BPA-exposed males (Fig. 3E). At adulthood (d 120), a sharp decrease in AR expression was observed in the ventral prostate periductal stroma of controls, with no significant differences observed between groups (Fig. 3E and Table 4).

Prostatic acid phosphatase immunostaining was restricted to the glandular epithelial cells of the proximal and distal ducts (Fig. 2, M–R, and Table 4). PAP was

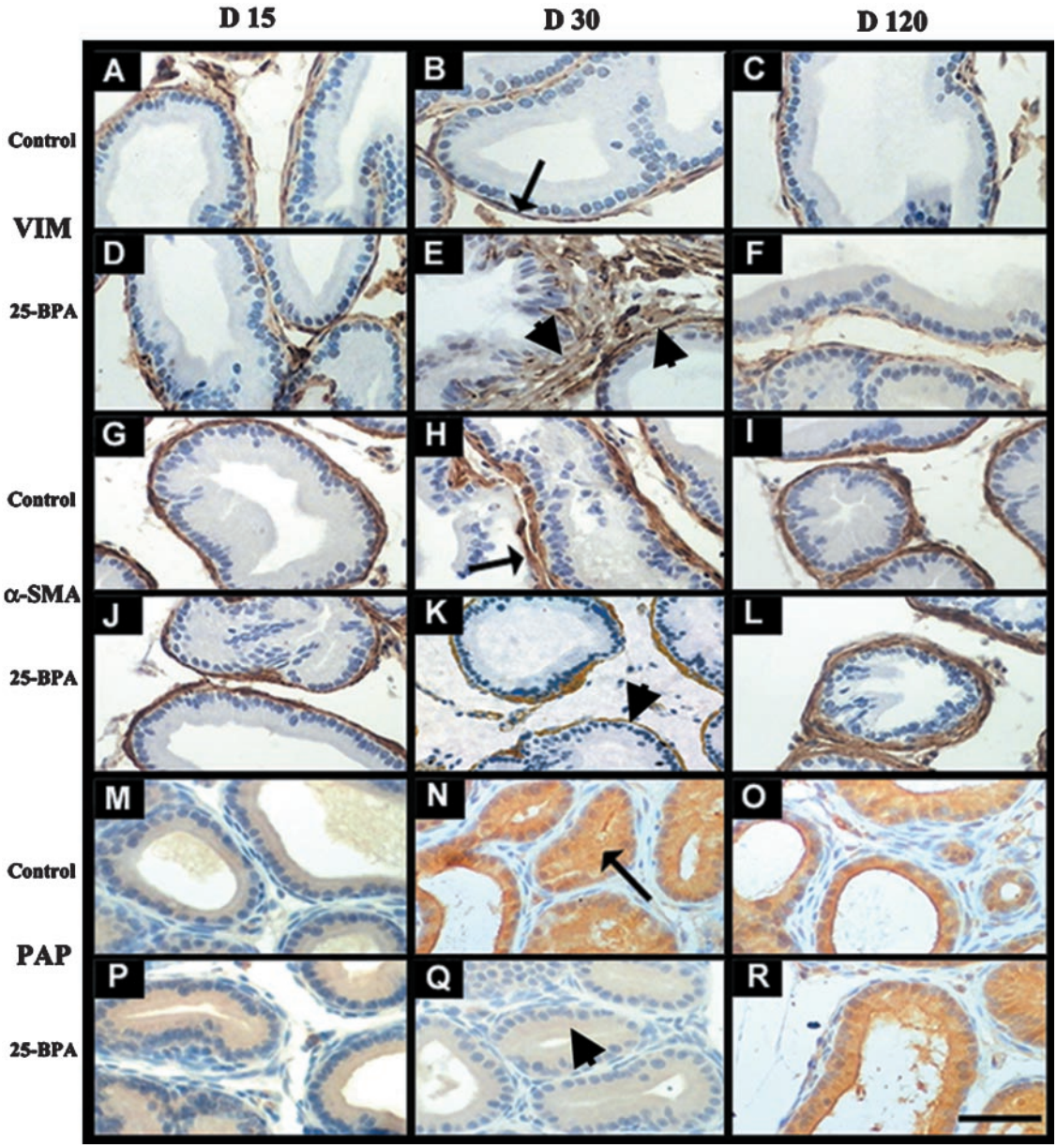

FIG. 2. Photomicrographs showing a transient histofunctional disruption of the rat ventral prostate after *in utero* exposure to BPA. Immunohistochemical evaluation of Vim, α-SMA, and PAP was performed at prepuberty (d 15), peripuberty (d 30), and adulthood (d 120). In control animals, the periductal immunostaining pattern for Vim was discontinuous in distal tips (B, *arrow*), whereas the α-SMA layer formed a continuous ring around the ducts (H, *arrow*). In BPA-exposed groups, the Vim-staining pattern (fibroblastic cells) was presented as a uniform, thick halo surrounding the ducts only on d 30 (E, *arrowhead*). In addition, on d 30, the α-SMA layer (smooth muscle cells) presented several discontinuities and was thinner (K, *arrowhead*) than in controls. No differences were observed in Vim and/or α-SMA expression patterns in the periductal stroma of d 15 and 120 rats. PAP is a cytoplasmic marker of epithelial cells (M–R). Although PAP expression was present throughout the entire cytoplasm, the apical region was more intensely stained than basal region (N, *arrow*). In control animals, there was a sharp increase in the percentage of PAP^+ cells, and in the staining intensity from d 15–30, which was sustained on d 120. In contrast, BPA-exposed animals did not exhibit this sharp increase on d 30 (Q, *arrowhead*); levels of PAP expression returned to control values by d 120. *Solid bar*, 50 μm.

expressed throughout the cytoplasm; however, staining was concentrated in the apical region. In controls there was a sharp increase in the percentage of PAP^+ cells and in the staining intensity from d 15–30 (Fig. 2, M *vs.* N), which was sustained on d 120 (Fig. 2O). In contrast, BPA-exposed animals did not exhibit this sharp increase on d 30 (Fig. 2Q), and levels of PAP expression returned to control values on d 120 (Figs. 2R and 3F).

Prenatal BPA exposure permanently up-regulates ERβ mRNA expression in the male POA

The relative expression of ERα and ERβ mRNAs in the POA and MBH of d 30 and 120 animals was evaluated by RT-PCR. Validation of the RT-PCR assays is summarized in Fig. 1. For amplification in the exponential phase of PCR, different numbers of cycles were tested for each mRNA. All linear correlation

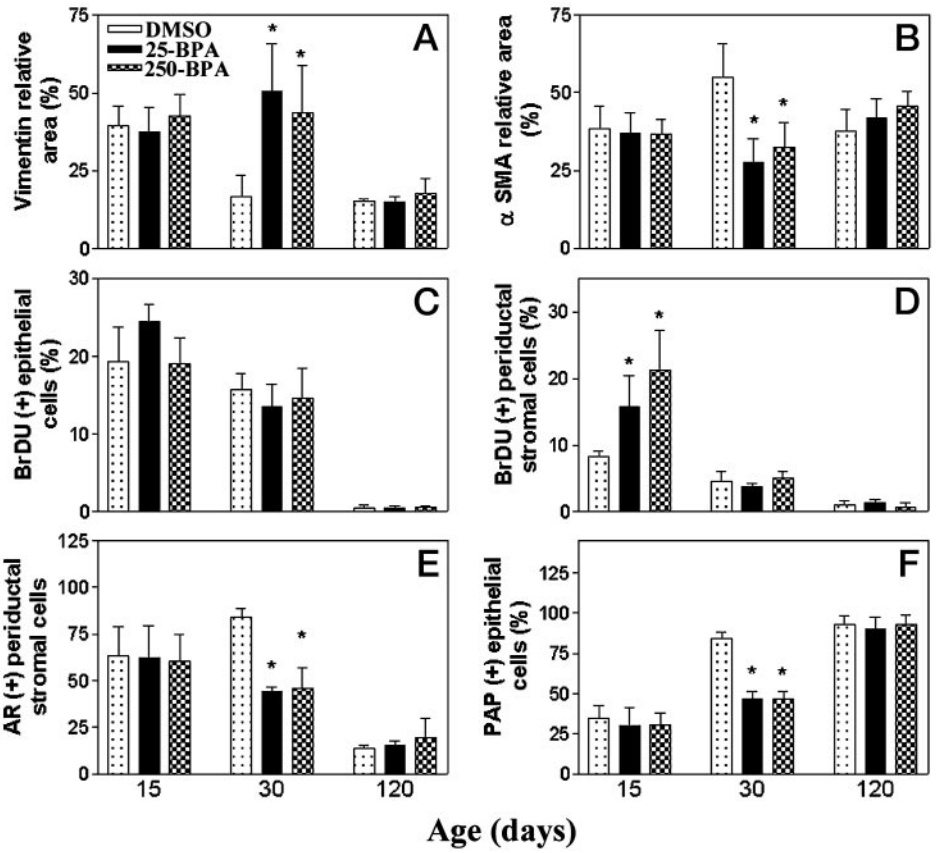

FIG. 3. Quantitative evaluation of histofunctional markers in the rat ventral prostate after *in utero* exposure to BPA. Prostate tissue was evaluated on different postnatal days: prepuberty (d 15), peripuberty (d 30), and adulthood (d 120). A, Relative area occupied by Vim^+ cells. In BPA-exposed groups, the area occupied by fibroblastic cells is larger than that in controls (only in d 30 rats). B, Relative area occupied by α-SMA^+ cells. Note that the α-SMA relative area is smaller in BPA-exposed rats than in controls on d 30. C and D, Percentage of BrdU incorporation into epithelial (C) and periductal stromal (D) cells. A higher BrdU index was obtained in the periductal stroma of BPA-exposed animals on d 15. E, AR expression in periductal stromal cells. On d 30, BPA-exposed groups exhibited a lower percentage of AR^+ stromal cells in the periductal ring than control rats. F, PAP expression in epithelial cells. On d 30, control animals exhibited a greater number of glandular epithelial PAP^+ cells than BPA-exposed groups. *Asterisks* indicate significant differences between DMSO (control) and 25-BPA- or 250-BPA-exposed rats ($P < 0.05$).

TABLE 4. Proliferation and differentiation markers in the rat ventral prostate prenatally exposed to BPA

Marker assessed	d 15			d 30			d 120		
	Control	25-BPA	250-BPA	Control	25-BPA	250-BPA	Control	25-BPA	250-BPA
Epithelial BrdU[1]	19.3 ± 4.3	24.3 ± 2.2	18.9 ± 3.3	15.7 ± 1.9	13.5 ± 2.8	14.6 ± 3.9	0.46 ± 0.3	0.44 ± 0.2	0.60 ± 0.2
Periductal BrdU[1]	8.33 ± 0.8^a	15.7 ± 4.6^b	21.2 ± 5.9^b	4.65 ± 1.3	3.76 ± 0.5	5.04 ± 0.9	1.00 ± 0.6	1.40 ± 0.4	0.75 ± 0.6
Vim relative area[2]	39.3 ± 6.5	37.4 ± 7.7	42.5 ± 6.7	16.6 ± 6.7^a	50.6 ± 15^b	43.8 ± 15^b	15.2 ± 0.6	14.7 ± 1.9	17.8 ± 4.5
αSMA relative area[2]	38.4 ± 7.1	37.1 ± 6.2	36.5 ± 5.0	54.8 ± 10^a	27.8 ± 7.5^b	32.4 ± 8.0^b	37.5 ± 7.1	41.7 ± 6.2	45.5 ± 5.1
Periductal AR[1]	63.3 ± 15	62.3 ± 17	60.6 ± 14	84.4 ± 4.1^a	44.1 ± 2.5^b	46.1 ± 10^b	13.5 ± 1.9	15.3 ± 2.3	19.6 ± 10
PAP[1]	34.5 ± 8.0	30.0 ± 11	30.5 ± 7.2	84.0 ± 4.1^a	46.5 ± 4.7^b	46.6 ± 4.8^b	93.0 ± 5.2	90.3 ± 7.1	93.0 ± 6.3
ERα[1]	NR	NR	NR	NR	NR	NR	NR	NR	NR

Results are expressed as percentage of positive cells ([1]) or as percentage of the periductal area occupied by the marker ([2]). Values are means ± SEM. NR, Negative results. *Different letters* indicate statistically significant differences for each marker between experimental groups ($P < 0.01$).

coefficients were greater than 0.97 between 32 and 38 cycles for ERα and ERβ, and between 16 and 22 cycles for L19 ribosomal protein. Optimization of RT-PCR conditions was achieved using total RNA from a pool of control and BPA-exposed rat hypothalami. Quantitative analysis revealed a strong linear relationship between the amount of starting RNA and the intensity of the generated PCR signals (Fig. 1).

ERα and ERβ expression were differentially modulated by BPA in the POA (Fig. 4, A and B). Both doses of BPA employed induced a significant increase in POA ERβ mRNA expression in peripubertal d 30 animals ($P < 0.01$; Fig. 4A). Overexpression of ERβ mRNA in the POA was maintained on d 120 of BPA-exposed males ($P < 0.01$; Fig. 4B), suggesting that this was a permanent effect. On the other hand, BPA treatment did not alter POA ERα mRNA expression levels, and neither ERα nor ERβ mRNA quantities were modified in the MBH of BPA-exposed groups in comparison with controls (Fig. 4, A and B).

Prenatal BPA exposure induces transient increases in T and PRL serum levels

Both control and BPA-exposed animals exhibited a gradual increase in PRL and T serum levels throughout the study period (Fig. 5, A and C). No differences in PRL serum levels were observed between prepubertal (d 15) males prenatally exposed to BPA and control animals. On d 30, BPA-exposed males exhibited significantly higher PRL levels compared with controls; however, no differences were observed between 25-BPA- and 250-BPA-exposed groups (Fig. 5A). Upon adulthood (d 120), PRL serum levels in BPA-exposed males returned to normal (Fig. 5A). LH serum levels did not differ significantly between BPA-exposed animals and controls on any of the postnatal days examined (Fig. 5B), although BPA-exposed groups exhibited a high degree of variability.

Low levels of T were detected on d 15 and d 30 in controls,

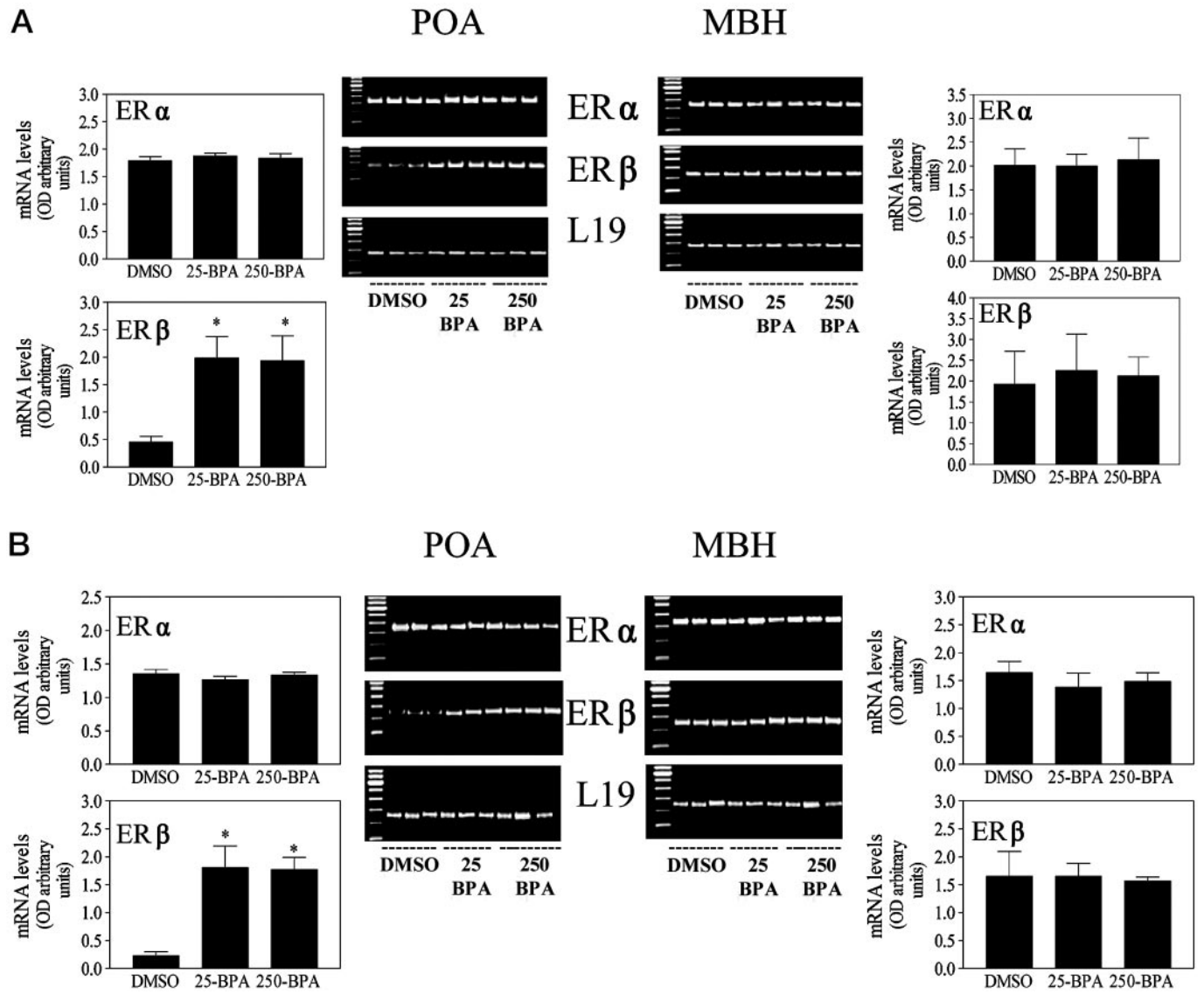

FIG. 4. Representative ethidium bromide-stained gel electrophoresis of ERα and ERβ mRNA expression in the POA and MBH from controls and *in utero* BPA-exposed rats studied on different days postnatally. *Bars* represent the mean ± SEM (n = 6–8) of relative expression levels of ERα and ERβ mRNAs. Relative expression levels were obtained by normalization of the absolute OD values of each target (ERα and ERβ) to those of the L19 signal. A, Relative expression levels of ERα and ERβ mRNAs in the POA (*left panel*) and MBH (*right panel*) of d 30 rats. A significant increase in ERβ mRNA expression occurs in the POA of BPA-exposed rats ($P < 0.01$), whereas no significant differences in ERα or ERβ mRNA levels were observed in the MBH. B, ERα and ERβ mRNA levels in the POA (*left panel*) and MBH (*right panel*) of d 120 rats. ERβ overexpression was maintained in the POA of *in utero* BPA-exposed males ($P < 0.01$); in contrast, no significant differences were observed in ERα and ERβ mRNA expression in the MBH in all groups examined.

whereas higher serum levels were observed in adult animals (d 120). Prenatal administration of BPA resulted in a transient, but significant, increase in serum T levels on d 15 in both BPA-exposed groups. This increase was not observed on d 30 and 120 postnatally (Fig. 5C).

Discussion

Data from recent reports have suggested that neonatal exposure to low doses of β-estradiol-3-benzoate can induce temporary changes in the reproductive tract of male rats (18, 19). These transient effects include an increase in the relative weight of the dorsal lobes in Sprague Dawley rats during the peripubertal period (18). These changes were not observed upon adulthood, suggesting a reversible process that could be triggered during certain estrogen-sensitive critical stages of development, such as the prenatal and neonatal periods (10, 15, 32). However, little is known about the ontogeny of the deleterious effects of low, environmentally relevant doses of xenoestrogens on rodent reproductive organs and related neuroendocrine circuits (33–36). The doses used in our experiments were lower than the NOAEL (no observed adverse effect levels) dose declared by the National Toxicology Program's report (2000) on low-dose endocrine disrupters for BPA in rats. Therefore, our results introduce novel information regarding BPA actions at concentrations that have been considered safe by traditional toxicological par-

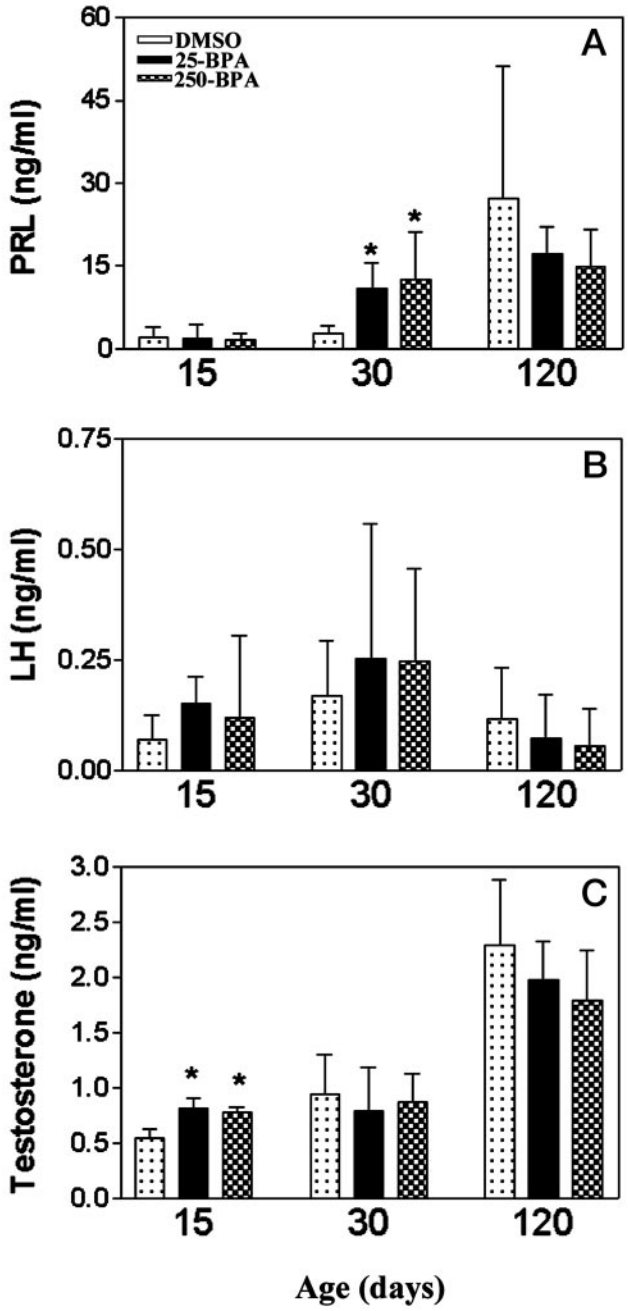

FIG. 5. Serum PRL (A), LH (B), and T (C) levels in control and BPA-exposed rats at different ages (d 15, 30, and 120). Values are the mean ± SD of plasma hormone levels from five to seven rats per group. *Asterisks* indicate significant differences between the DMSO- and 25-BPA- or 250-BPA-exposed rats ($P < 0.05$).

adigms. Our data reveal that prenatal exposure to BPA increased ERβ mRNA expression in the POA of male rats, and this effect appears to be permanent, as it was observed in both peripubertal and adult animals. However, no differences were observed in ERβ mRNA expression in the MBH, suggesting the existence of specific anatomical regions in the anterior rat brain that are sensitive to the action of BPA. In addition, no changes were observed in ERα mRNA expression in both brain regions analyzed. Our results are in agreement with previous findings (13) showing no alterations in ERα and ERβ mRNA expression in the medial hypothalamus of neonatally BPA- and octylphenol-exposed male rats. However, this is the first report showing a permanent increase in ERβ mRNA expression in the POA of prenatally BPA-exposed rats. Previous reports have demonstrated that ERβ mRNA expression is sexually dimorphic in several nuclei of the rat POA and that orchidectomy of male neonates completely reverses the sexual phenotype of ERβ expression in the anteroventral periventricular nucleus (37). *In utero* BPA exposure could disrupt ERβ expression in sexual dimorphic structures, either modifying the total number of ERβ-positive cells or affecting neuronal migration patterns during critical periods of brain maturation (38, 39). It has been shown that ERα expression in the male rat brain is necessary to maintain normal sexual behavior patterns such as mounting or ejaculation (40). Because ERβ can repress transcriptional activity of ERα through the formation of ERα/ERβ heterodimers (41), and *in utero* BPA exposure reduces both the motivation to explore and anxiety in male pups (42), a possible relationship between the overexpression of ERβ in the POA and abnormal sexual behavior should not be ruled out. It has been established that LH-releasing hormone (LHRH) neurons of the POA in male rats also coexpress ERβ mRNA/protein (43). Moreover, repression of the LHRH promoter by estradiol has been shown in LHRH-producing GT1 cells (44). In our experimental setting, prenatal exposure to BPA resulted in a marked increase in POA ERβ mRNA expression levels; therefore, it is reasonable to hypothesize that prenatal exposure to xenoestrogens may disrupt the sensitive negative feedback mechanisms normally regulating LHRH secretion. In the mouse, ERβ expression in the anterior hypothalamus from the late gestational to the prepubertal period is higher in males, which exhibit high levels of locally produced estrogen after aromatization of testosterone (45). Thus, increased expression of the ERβ subtype in the POA may contribute to a defective maturation of LHRH control circuits. These events could explain the higher T levels observed in BPA-exposed males during peripubertal periods. On the other hand, constitutive *trans*-activation of the vasopressin gene promoter by the unliganded ERβ has been demonstrated using *in vitro* experiments (46). A similar interaction between high levels of ERβ detected in BPA-treated males with the LHRH gene also cannot be ruled out, arising as an LHRH mRNA transcription control mechanism in a ligand-independent fashion (43, 46). Confirmation of this hypothesis awaits further investigation.

Hyperprolactinemia is a well-established consequence of neonatal xenoestrogen exposure in estrogen-sensitive rats such as the Fisher 344 strain (12–14). However, little is known about the persistence of this phenomenon beyond the peripubertal age. Our results, using a Wistar-derived strain, indicate that the hyperprolactinemia is transient and is evident only in d 30 rats. It is not clear whether the xenoestrogen-induced hyperprolactinemia affects the onset of puberty and/or fertility in male rats. These issues will need to be addressed in future experiments.

In the present study, we did not observe significant differences in ventral prostate weights, AGD, or pups' body weights between BPA-exposed and control animals. These

results are in accordance with previous studies that used a similar range of BPA doses (47). However, we demonstrated in a previous study (15) that prenatal exposure to BPA alters the differentiation pattern of the periductal stromal cells in the rat ventral prostate on postnatal d 30. These results taken together suggest that differences in organ weight-related end points would not have enough sensitivity to significantly evaluate the effects of low doses of BPA. In BPA-exposed groups, the presence of a thick layer of $Vim^+/\alpha\text{-}SMA^-$ cells in the periductal zone contrasts with the multicellular smooth muscle $Vim^-/\alpha\text{-}SMA^+$ layer observed in controls. Herein, we demonstrated that the presence of a thicker layer of fibroblasts in the periductal stroma on d 30 is the consequence of a modified proliferative status during early development (d 15). Contemporary with this disruption of cellular dynamics in BPA-exposed rats, increased T serum levels were observed. A cause-effect relationship between higher T serum levels and the modified proliferative indexes in the periductal stroma cannot be ruled out. Previous work has shown that ERα expression is confined to mesenchymal cells only during early development of ventral prostate (48). In agreement with this study and confirming our previous report (15), control groups were negative for ERα in both epithelial and stromal compartments, and BPA did not modify this pattern of expression. Decreased PAP expression observed in BPA-exposed groups could be mediated by either a direct effect of BPA on the columnar epithelial cells or through an indirect consequence of primary events occurring in the stroma (16, 17, 49). Stromal signals are believed to be critical in determining the decision of epithelial cells to undergo proliferation, apoptosis, or differentiation. The decreased AR expression observed in the periductal stromal cells on d 30 may affect the androgen-signaling pathway resulting in decreased PAP expression, although a direct effect of BPA on the epithelial cells also cannot be ruled out. During adulthood, no differences were observed in the periductal stromal architecture. Prostatic acid phosphatase expression in columnar epithelial cells during adulthood was increased in all groups analyzed, and the differences observed on d 30 were not present on d 120. In addition, functional relationships have been established between hyperprolactinemia and prostatic tissue disorders in rats (14). As the histoarchitectural and functional changes that were observed in the ventral prostates of BPA-exposed rats are temporally associated with elevated serum PRL levels, further studies will need to be undertaken to elucidate the possible relationships between these phenomena. The transient characteristic of the changes observed in the ventral prostates and in T and PRL serum levels could be the result of early organizational effects that are associated with the actions of exogenous or endogenous estrogens during organogenesis and development (1). Our hypothesis is that organizational effects induced by low doses of xenoestrogens during early development stages could be expressed during critical periods such as puberty, when the hormonal milieu exhibits a high degree of variation, and compensatory mechanisms (such as negative feedback systems) are not fully developed.

In summary, *in utero* exposure to environmentally relevant levels of BPA increased ERβ mRNA expression in the anterior hypothalamic structures of the pubertal male rat, and this overexpression persisted into adulthood. In contrast, prenatal exposure to BPA transiently affects the ventral prostate of peripubertal rats by decreasing AR expression, altering proliferative activity in the periductal stromal cells, and decreasing PAP expression. All of these morphological and functional changes that were observed in the ventral prostate on d 30 were transient, as they were not observed during adulthood. In addition, other transient effects observed were increases in T and PRL serum levels in prepubertal BPA-exposed rats, whereas no differences were detected during adulthood. A close time dependence of the biological responses to xenoestrogens during development together with the differential estrogen sensitivity of the various rat strains employed and/or end points examined could explain at least in part the extraordinary variability in the *in vivo* results obtained to date regarding endocrine disrupters (1, 10, 47, 50, 51).

The findings reported in this study support the hypothesis that environmental xenoestrogen exposure may be associated with low seminal quality and other male reproductive dysfunctions that affect human fertility (2, 3).

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References

1. **McLachlan JA** 2001 Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr Rev 22:319–341
2. **Oliva A, Giami A, Multigner L** 2002 Environmental agents and erectile dysfunction: a study in a consulting population. J Androl 23:546–550
3. **Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, MacLachlan JA, Meyer OM, Muller J, Rajperts-De Meyts E, Scheike T, Sharpe R, Sumpler J, Skakkebaek NE** 1996 Male reproductive health and environmental xenoestrogens. Environ Health Perspect 104:741–803
4. **Brotons JA, Olea-Serrano MF, Villalobos M, Olea N** 1994 Xenoestrogens released from lacquer coating in food cans. Environ Health Perspect 103: 608–612
5. **Biles JE, McNeal TP, Begley TH, Hollifield HC** 1997 Determination of Bisphenol-A in reusable polycarbonate food-contact plastics and migration to food simulating liquids. J Agric Food Chem 45:3541–3544
6. **Olea N, Pulgar R, Perez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C** 1996 Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 104:298–305
7. **Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y** 2002 Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 17:2839–2841
8. **Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I** 2002

Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 110:A703–A707
9. **Welshons WV, Nagel SC, Thayer KA, Judy BM, Vom Saal FS** 1999 Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice. Toxicol Ind Health 15:12–25
10. **Gupta C** 2000 Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc Soc Exp Biol Med 224:61–68
11. **Rubin BS, Murray MK, Damassa DA, King JC, Soto AM** 2001 Perinatal exposure to low doses of bisphenol-A affects body weight, patterns of estrous cyclicity and plasma LH levels. Environ Health Perspect 109:675–680
12. **Steinmetz R, Brown NG, Allen DL, Bigsby RM, Ben-Jonathan N** 1997 The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo.* Endocrinology 138:1780–1786
13. **Khurana S, Ranmal S, Ben-Jonathan N** 2000 Exposure of newborn male and female rats to environmental estrogens: delayed and sustained hyperprolactinemia and alterations in estrogen receptor expression. Endocrinology 141:4512–4517
14. **Stoker TE, Robinette CL, Britt BH, Laws SC, Cooper R** 1999 Prepubertal exposure to compounds that increase prolactin secretion in the male rat: effects on the adult prostate. Biol Reprod 61:1636–1643
15. **Ramos JG, Varayoud J, Sonnenschein C, Soto AM, Muñoz-de-Toro M, Luque EH** 2001 Prenatal exposure to low doses of bisphenol A alters the periductal stroma and glandular cell function in the rat ventral prostate. Biol Reprod 65:1271–1277
16. **Chang WY, Wilson MJ, Birch L, Prins GS** 1999 Neonatal estrogen stimulates proliferation of periductal fibroblasts and alters the extracellular matrix composition in the rat prostate. Endocrinology 140:405–415
17. **Habermann H, Ghang WY, Birch L, Mehta P, Prins GS** 2001 Developmental exposure to estrogens alters epithelial cell adhesion and gap junction proteins in the adult rat prostate. Endocrinology 142:359–369
18. **Putz O, Schwartz CB, Kim S, LeBlanc GA, Cooper RL, Prins GS** 2001 Neonatal low- and high-dose exposure to estradiol benzoate in the male rat. I. Effects on the prostate gland. Biol Reprod 65:1496–1505
19. **Putz O, Schwartz CB, LeBlanc GA, Cooper RL, Prins GS** 2001 Neonatal low- and high-dose exposure to estradiol benzoate in the male rat. II. Effects on the prostate gland. Biol Reprod 65:1506–1517
20. **Paxinos G, Watson C** 1997 The rat brain in stereotaxic coordinates. Compact 3rd ed. San Diego: Academic Press
21. **Muñoz-de-Toro M, Maffini M, Kass L, Luque EH** 1998 Proliferative activity and steroid hormone receptor status in male breast carcinoma. J Steroid Biochem Mol Biol 67:333–339
22. **Kass L, Varayoud JG, Ortega HH, Muñoz-de-Toro MM, Luque EH** 2000 Detection of bromodeoxyuridine in formalin-fixed tissue. DNA denaturation following microwave or enzymatic digestion pretreatment is required. Eur J Histochem 44:185–191
23. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
24. **Tena-Sempere M, Navarro J, Pinilla L, González L, Huhtaniemi I, Aguilar E** 2000 Neonatal exposure to estrogen differentially alters estrogen receptor α and β mRNA expression in rat testis during postnatal development. J Endocrinol 165:345–357
25. **Koike S, Saka M, Muramatsu M** 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 15:2499–2513
26. **Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsso S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930.
27. **Chang X-L, Lin A, McNally J, Pelleg D, Meyuha O, Wool Y** 1987 The primary structure of rat ribosomal protein L19. J Biol Chem 262:1111–1115
28. **Lux-Lantos V, Becu-Villalobos D, Bianchi M, Rey-Roldan E, Chamson-Reig A, Pignataro O, Libertun C** 2001 GABA(B) receptors in anterior pituitary cells. Mechanism of action coupled to endocrine effects. Neuroendocrinology 73: 334–343
29. **Luque EH, Muñoz-de-Toro M, Smith PF, Neill JD** 1986 Subpopulations of lactotropes detected with the reverse hemolytic plaque assay show differential responsiveness to dopamine. Endocrinology 118:2120–2124
30. **Suescun MO, Gonzalez SI, Chiauzzi VA, Calandra RS** 1985 Effects of induced hypoprolactinemia on testicular function during gonadal maturation in the rat. J Androl 6:77–82
31. **Siegel S** 1956 Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill
32. **Timms BG, Petersen SL, Vom Saal FS** 1999 Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. J Urol 161:1694–1701
33. **Vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV** 1998 A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239–260
34. **Colerangle JB, Roy D** 1997 Profound effects of the weak environmental estrogen-like chemical bisphenol A on the growth of the mammary gland of Noble rats. J Steroid Biochem Mol Biol 60:153–160
35. **Sheehan DM** 2000 Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: initial results confirmed. Proc Soc Exp Biol Med 224:57–60
36. **Funabashi T, Kawaguchi M, Kimura F** 2001 The endocrine disrupters butyl benzyl phthalate and bisphenol A increase the expression of progesterone receptor messenger ribonucleic acid in the preoptic area of adult ovariectomized rats. Neuroendocrinology 74:77–81
37. **Orikasa C, Kondo Y, Hayashi S, McEwen BS, Sakuma Y** 2002 Sexually dimorphic expression of estrogen receptor β in the anteroventral periventricular nucleus of the rat preoptic area: Implication in luteinizing hormone surge. Proc Natl Acad Sci USA 99:3306–3311
38. **Tobet SA, Henderson RG, Whiting PJ, Sieghart W** 1999 Special relationship of γ-aminobutyric acid to the ventromedial nucleus of the hypothalamus during embryonic development. J Comp Neurol 405:88–98
39. **Dellovade TL, Young M, Ross EP, Henderson R, Caron K, Parker K, Tobet SA** 2000 Disruption of the gene encoding SF-1 alters the distribution of hypothalamic neuronal phenotypes. J Comp Neurol 423:579–589
40. **Ogawa S, Lubahn DB, Korach KS, Pfaff DW** 1997 Behavioral effects of estrogen receptor gene disruption in male mice. Proc Natl Acad Sci USA 94:1476–1481
41. **Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA** 2000 Estrogen receptor (ER) β, a modulator of ER α in the uterus. Proc Natl Acad Sci USA 97:5936–5941
42. **Farabollini F, Porrini S, Dessi-Fulgheri F** 1999 Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol Biochem Behav 64:687–694
43. **Hrabovszky E, Steinhauser A, Barabás K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z** 2000 Estrogen receptor-β immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 142:3261–3264
44. **Roy D, Angelini NL, Belsham DD** 1999 Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-α (ERα)- and ERβ-expressing GTI-7 GnRH neurons. Endocrinology 140:5045–5053
45. **Karolczak M, Beyer C** 1998 Developmental sex differences in estrogen receptor-β mRNA expression in the mouse hypothalamus/preoptic region. Neuroendocrinology 68:229–234
46. **Shapiro RA, Xu C, Dorsa DM** 2000 Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor α and β. Endocrinology 141:4056–4064
47. **Tinwell H, Haseman J, Lefevre PA, Wallis N, Ashby J** 2002 Normal sexual development off two strains of rats exposed *in utero* to low doses of bisphenol A. Toxicol Sci 68:339–348
48. **Prins GS, Birch L** 1997 Neonatal estrogen exposure up-regulates estrogen receptor expression in the developing an adult rat prostate lobes. Endocrinology 138:1801–1809
49. **Grossfeld GD, Hayward SW, Tlsty TD, Cunha GR** 1998 The role of stroma in prostatic carcinogenesis. Endocrine-Related Cancer 5:253–270
50. **Kwon S, Stedman DB, Elswick BA, Cattley RC, Welsch F** 2000 Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats expose to bisphenol A during prenatal and postnatal development. Toxicol Sci 55:399–406
51. **Goloubkova T, Ribeiro MFM, Rodrigues LP, Cecconello AL, Spritzer PM** 2000 Effects of xenoestrogen bisphenol A on uterine and pituitary weight, serum prolactin levels and immunoreactive prolactin cells in ovariectomized Wistar rats. Arch Toxicol 74:92–98