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The Industrial Chemical Bisphenol A (BPA) Interferes with Proliferative Activity and Development of Steroidogenic Capacity in Rat Leydig Cells[1]

Manjunatha K. Nanjappa,[3] Liz Simon,[4] and Benson T. Akingbemi[2,3]

[3]*Department of Anatomy, Physiology, and Pharmacology, Auburn University, Auburn, Alabama*
[4]*Department of Biomedical Sciences, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Alabama*

ABSTRACT

?2 **The presence of bisphenol A (BPA) in consumer products has raised concerns about potential adverse effects on reproductive health. Testicular Leydig cells are the predominant source of the male sex steroid hormone testosterone, which supports the male phenotype. The present report describes the effects of developmental exposure of male rats to BPA by gavage of pregnant and lactating Long-Evans dams at 2.5 and 25 μg/kg body weight from Gestational Day 12 to Day 21 postpartum. This exposure paradigm stimulated Leydig cell division in the prepubertal period and increased Leydig cell numbers in the testes of adult male rats at 90 days. Observations from in vitro experiments confirmed that BPA acts directly as a mitogen in Leydig cells. However, BPA-induced proliferative activity in vivo is possibly mediated by several other factors, such as 1) protein kinases (e.g., mitogen-activated protein kinases or MAPK), 2) growth factor receptors (e.g., insulin-like growth factor 1 receptor-beta and epidermal growth factor receptors), and 3) the Sertoli cell-secreted anti-Mullerian hormone (also called Mullerian inhibiting substance). On the other hand, BPA suppressed protein expression of the luteinizing hormone receptor (LHCGR) and the 17beta-hydroxysteroid dehydrogenase enzyme (HSD17B3), thereby decreasing androgen secretion by Leydig cells. We interpret these findings to mean that the likely impact of deficits in androgen secretion on serum androgen levels following developmental exposure to BPA is alleviated by increased Leydig cell numbers. Nevertheless, the present results reinforce the view that BPA causes biological effects at environmentally relevant exposure levels and its presence in consumer products**
?3 **potentially has implication for public health.**

Developmental biology, Environmental contaminants and toxicants, Leydig cells, Sertoli cells, Toxicology

INTRODUCTION

Bisphenol A (BPA) is used in the manufacture of a variety of consumer products, including polycarbonate plastics, epoxy resins, electronics, medical equipment, and dental sealants [1, 2], and qualifies as one of the world's leading volume-production chemicals with an annual increase in demand of 6%–10% [3]. This chemical is able to leach from containers into foods when heated and from dental sealants into the saliva of patients after application and can cross the human placenta into fetal tissues [4, 5]. Interestingly, it had been determined that 90% of the population in the United States has BPA in their urine samples [6, 7], implying widespread exposure of the population to this compound in the environment. For example, the levels of BPA were determined to be 0.3–18.9 ng/mL in maternal plasma, whereas the levels were 0.2–9.2 in fetal plasma and 0.28–0.97 in breast milk [5, 8]. In the adult U.S. population, the mean urinary BPA level was determined to be about 2.5 ng/mL [6], and the concentration of unconjugated or free BPA in blood was approximately 1 ng/mL [9].

The biological actions of BPA are thought to be due mostly to its estrogenic properties. In this regard, the male reproductive tract, including testicular Leydig cells, expresses high levels of estrogen receptors (ESR1, ESR2) along with androgen receptors (ARs) [10]. Although BPA is thought to have a higher affinity for ESR2, it is able to induce both ESR1- and ESR2-mediated transcriptional activity with comparable efficacy in several tissues [1, 11]. In addition, BPA may elicit biological responses by activation of kinase cascades (e.g., MAPK [12]) and by exhibiting antiandrogenic [13, 14] and/or antithyroid activity [15]. Other reports have suggested that BPA increased bioavailability of sex steroid hormones by disrupting metabolic degradation [16–18].

We observed previously that exposure of male rats to BPA at ~2.5 μg/kg/day from Gestational Day (GD) 12 to Postnatal Day (PND) 21 did not affect serum testosterone (T) levels but decreased Leydig cell T production and intratesticular T concentrations in adult animals [19]. The present report extends our initial findings and is focused on postnatal differentiation of Leydig cells in male rats perinatally exposed to BPA. In the rat, Leydig cell development progresses through three stages and is defined by two features: proliferation and steroid hormone secretion. Early in the neonatal period, mesenchymal Leydig cell precursors differentiate into highly proliferative progenitor Leydig cells (PLCs) from PND 14 to 21 and then into immature Leydig cells (ILCs) between PND 21 and 35. Although ILCs are less able to divide than PLCs, they gradually transform into fully steroidogenic adult Leydig cells (ALCs) by PND 56. ALCs are devoid of any mitotic capacity but have a 150- and 5-fold greater capacity for T production than PLCs and ILCs [20].

Importantly, serum androgen levels derive from both steroidogenic capacity and the number of Leydig cells in the testes. Therefore, we hypothesized that disparities in Leydig cell T production and serum androgen levels in BPA-exposed animals [19] are likely due to changes in proliferative activity early in development that affect Leydig cell numbers. In this regard, a primary feature of Leydig cell development is the

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[2]Correspondence: Benson T. Akingbemi, PhD, Department of Anatomy, Physiology, and Pharmacology, Auburn University, Auburn, AL 36849. E-mail: akingbt@auburn.edu

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TABLE 1. Antibodies used in immunoblotting procedures.

Antibody	Dilutions	Host species	Catalog no.	Company
PCNA	250	Mouse monoclonal	sc-53409	Santa Cruz Biotechnologies
Cyclin D3	250	Rabbit polyclonal	sc-182	Santa Cruz Biotechnologies
ESR1	1000	Mouse monoclonal	ab 2746	Abcam Inc.
AR	1000	Rabbit polyclonal	sc-185	Santa Cruz Biotechnologies
LHCGR	1000	Rabbit polyclonal	sc-25828	Santa Cruz Biotechnologies
STAR	2000	Rabbit polyclonal	Sc-25806	Santa Cruz Biotechnologies
CYP11A1	250	Goat polyclonal	sc-18043	Santa Cruz Biotechnologies
CYP17A1	10000	Goat polyclonal	sc-46081	Santa Cruz Biotechnologies
HSD17B	500	Goat polyclonal	sc-66415	Santa Cruz Biotechnologies
HSD3B	1000	Rabbit polyclonal	sc-28206	Santa Cruz Biotechnologies
β-actin	2000	Goat polyclonal	sc-1616	Santa Cruz Biotechnologies
EGFR	500	Rabbit polyclonal	sc-03	Santa Cruz Biotechnologies
IGF1RB	500	Rabbit polyclonal	Sc-713	Santa Cruz Biotechnologies
p-MAPK3/1	500	Mouse monoclonal	Sc-7383	Santa Cruz Biotechnologies
MAPK3/1	2000	Rabbit polyclonal	Sc-93	Santa Cruz Biotechnologies
AMHR2	1000	Rabbit polyclonal	Sc-67287	Santa Cruz Biotechnologies
AMH	500	Goat polyclonal	Sc-6886	Santa Cruz Biotechnologies

occurrence of active waves of mitosis in the prepubertal period, and it is conceivable that mitogens utilize a combination of mechanisms to regulate Leydig cell division. For example, cell cycle proteins, which support DNA synthesis (e.g., proliferating cell nuclear antigen [PCNA]) or the transition from the G1 to S phase of the cell cycle (e.g., cyclin D3), are expressed at higher levels in highly proliferative PLCs than in ILCs and ALCs [21, 22]. Similarly, a physiological role for MAPK3/1 in the regulation of Leydig cell division was indicated by measurement of greater p-MAPK3/1 levels in PLCs than in ILCs and ALCs [23] and observations showing that Leydig cell-specific deletion of MAPK3/1 resulted in reduced Leydig cell numbers in mice [24]. Altogether, these findings imply that the levels of PCNA, cyclin D3, and p-MAPK3/1 protein expression are putative markers of Leydig cell division.

Moreover, there is evidence that sex steroids regulate Leydig cell mitosis because Leydig cell numbers were decreased in the testicular feminized mice (*tfm*) compared to the wild type, implying that ARs mediate mitogenic signals in Leydig cells [25]. Several reports have indicated that a primary target for estrogen action in the testis is the Leydig cell because the natural estrogen 17β-estradiol inhibited proliferation and regeneration of Leydig cells in male rats treated with the Leydig cell toxin ethane dimethylsulfonate (EDS) [26]. Also, Leydig cells of several mammalian species express growth factor receptors, such as insulin-like growth factor 1 receptors (IGF1RB) and epidermal growth factor receptors (EGFR), which are known to mediate estrogenic activity and regulate proliferative activity in Leydig cells [21, 27–32].

Given the physiological requirement for cell-cell interactions and the possibility that chemicals with estrogenic activity act simultaneously in multiple testicular cells, we postulate that paracrine signaling molecules have a role in mediating BPA effects. For example, the anti-Mullerian hormone (AMH) is a glycoprotein secreted by Sertoli cells to induce regression of Mullerian ducts during the process of sexual differentiation [33]. Interestingly, receptors for AMH are present in Leydig cells (AMHR2), and AMH was found to suppress Leydig cell regeneration in EDS-treated male rats [34, 35]. On the other hand, Leydig cell hyperplasia was evident in *Amh* knockout mice compared to the wild type [36]. These observations demonstrate that the AMH acts as a negative regulator of Leydig cell division. It is likely that BPA regulates expression of the *Amh* gene, which possesses estrogen response elements (EREs) in its promoter region [37, 38].

With the approach of puberty, development of steroidogenesis in Leydig cells is differentially regulated to optimize T production capacity during the transition from PLCs to ALCs. Therefore, LH receptors (LHCGR) and steroidogenic enzymes, including cytochrome P450 side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B), cytochrome P45017α-hydroxylase/$_{C17/20}$-lyase (CYP17A1), and

TABLE 2. Pregnancy outcome and reproductive parameters in male rats exposed to BPA.*

	Dose of BPA (per kg/day)		
Parameters	0	2.5 μg	25 μg
Litter size (no. of pups)[a]	12.35 ± 0.57	11.28 ± 0.59	10.92 ± 0.43
Pup sex ratio (male:female)[a]	1.38 ± 0.24	1.2 ± 0.13	1.08 ± 0.19
Body weights (g, PND 1)[a]	6.15 ± 0.16	6.41 ± 0.11	6.42 ± 0.08
Body weights (g, PND 7)[a]	12.18 ± 0.43	13.3 ± 0.41	13.08 ± 0.21
Body weights (g, PND 21)[b]	50.28 ± 0.70	51.46 ± 0.82	52.59 ± 0.62
Paired testis weights (g, PND 21)[b]	0.21 ± 0.00	0.22 ± 0.00	0.23 ± 0.00
Body weights (g, PND 35)[c]	123.64 ± 2.57	131.76 ± 1.99	123.42 ± 2.62
Paired testis weights (g, PND 35)[c]	1.09 ± 0.03	1.11 ± 0.07	1.07 ± 0.04
Body weights (g, PND 90)[d]	437.75 ± 13.94	446 ± 11.13	430.42 ± 0.4
Paired testis weights (g, PND 90)[d]	3.24 ± 0.32	3.34 ± 0.11	3.65 ± 0.09

* BPA exposure was achieved by maternal gavage from GD 12 to PND 21.
[a] Values were based on the litter as a unit of measurement (n = 14).
[b–d] Values were based on [b]n = 28–30, [c]n = 14, and [d]n = 8–10 from each group.

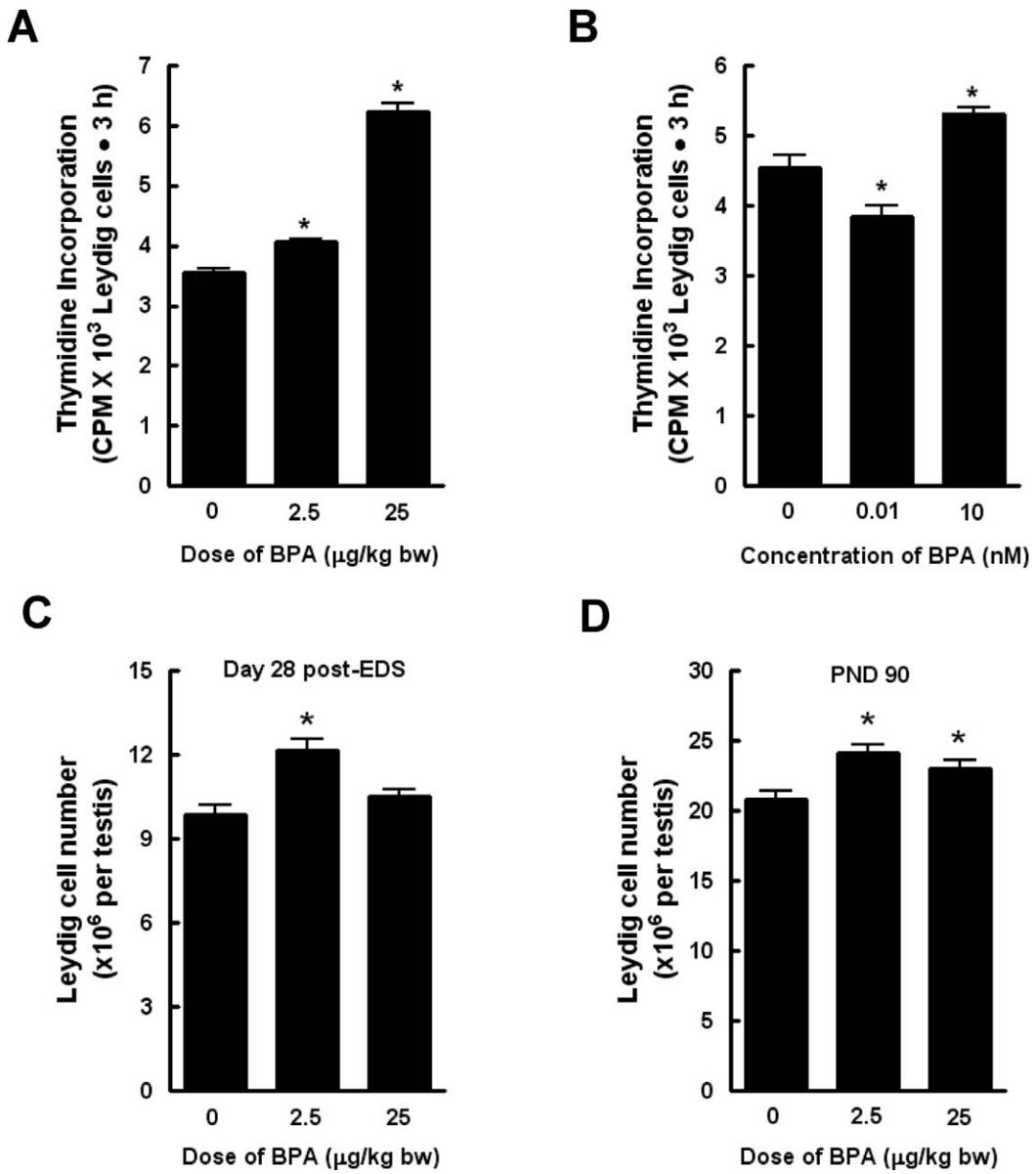

FIG. 1. Proliferative activity was assessed in vivo and in vitro using PLCs isolated and pooled from 28–30 male rats per treatment group at the end of perinatal BPA exposure on Day 21 postpartum (**A**) and BPA-free PLCs that were incubated in culture media containing BPA and ovine LH (10 ng/mL) for 18 h (**B**). Proliferative activity was determined by [^{3}H] thymidine incorporation assays performed in quadruplicate or greater followed by scintillation counting. Leydig cell numbers in the testes were enumerated by stereology 28 days after EDS administration to 60-day-old male rats exposed to BPA in the perinatal period (five animals per treatment group, PND 88; **C**). Leydig cells were also enumerated in testes of control and 90-day-old BPA-exposed male rats without EDS treatment (three animals per group; **D**). Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLCs, progenitor Leydig cells at 21 days of age; EDS, ethane dimethyl sulfonate; $^*P < 0.05$ compared to control.

17β-hydroxysteroid dehydrogenase type 3 (HSD17B3), are expressed at higher levels in ALCs than in ILCs and PLCs [20]. Because ALCs produce T required for spermatogenesis and maintenance of male secondary sexual characteristics, disruption of Leydig cell division and/or T biosynthesis due to activity of exogenous estrogens may be a factor in the causation of reproductive tract anomalies attributed to the action of environmental chemicals [2, 39]. In the present study, we asked whether exposure of male rats to BPA alters proliferative activity and Leydig cells numbers and/or affect T production capacity. In addition, we attempted to identify the cellular and molecular mechanisms of BPA action in Leydig cells.

MATERIALS AND METHODS

Animals

All experimental and euthanasia procedures were performed in accordance with a protocol approved by Auburn University Institutional Animal Care and Use Committee and recommendations of the panel on Euthanasia of the American Veterinary Medical Association. Time-bred pregnant Long-Evans dams at GD 6 were obtained from Harlan-Heklad and were allowed to acclimatize for 5–6 days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing Facility. Pregnant and nursing dams were housed one per cage, whereas weanling rats were kept in groups of two to four, depending on age and size. Animals were kept on a 12L:12D cycle, with ambient temperature of 68°F–74°F, and were provided feed and water ad

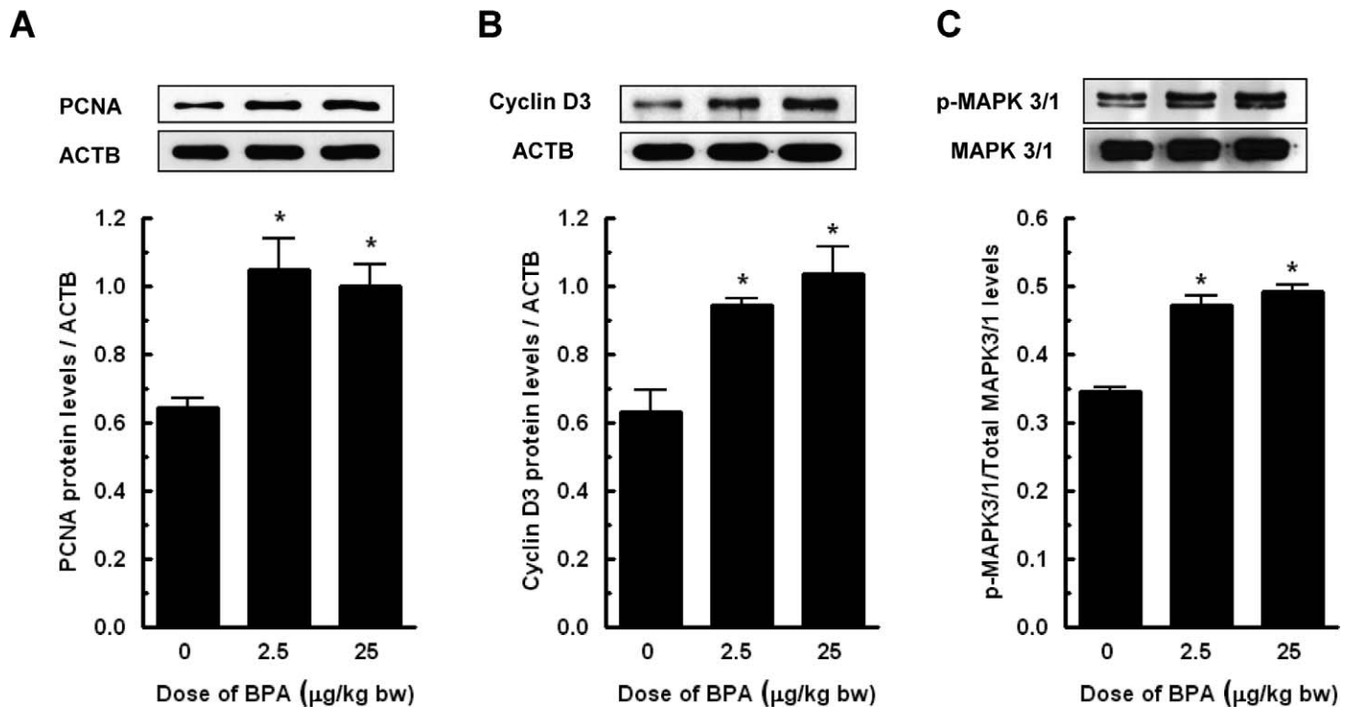

FIG. 2. At termination of perinatal BPA exposure on Day 21 postpartum, PLCs were isolated and pooled from 28–30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of proliferating cell nuclear antigen (PCNA; **A**), cyclin D3 (**B**), and activated (i.e., phosphorylated) extracellular regulated kinase (p-MAPK3/1; **C**). PCNA and cyclin D3 levels were normalized to β-actin (ACTB), while p-MAPK3/1 levels were normalized to total or inactive MAPK3/1 levels. Data represent results from densitometric analysis of at least four Western blots. PCNA, 36 kDa; cyclin D3, 35 kDa; p-MAPK3/1 and p-MAPK3/1, 42/44 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLCs: progenitor Leydig cells at 21 days of age. $*P < 0.05$ versus control.

libitum. However, standard rodent diets typically contain significant amounts of phytoestrogens (genistin and daidzin) [40], which have the potential to modulate endocrine responses in toxicological studies [41]. Also, use of polycarbonate cages may result in the leaching of BPA into the environment and alter experimental outcomes [42, 43]. In order to minimize background exposure of animals to estrogenic compounds, rats were fed on Teklad Global Soy Protein-Free Extruded Rodent Diet (#2020X; Harlan-Heklad) and housed in polypropylene cages with glass water bottles. Assignment of rats to groups was done by body weight randomization to ensure equal weight distribution.

Experimental Protocol

Pregnant dams were gavaged once a day with the olive oil vehicle or BPA (catalog no. 14939 Fluka; Sigma-Aldrich) in the oil vehicle at 2.5 or 25 μg/kg body weight (BW) from GD 12 through weaning at PND 21, that is, during the perinatal period (n = 14). Pregnant and nursing animals were weighed at 48-h intervals, and average body weights in each group were used to calculate BPA dosage. All dams carried pregnancy to full term, and pregnancy outcome, including litter size and pup weight, was assessed on the date of birth, which was designated as PND 1 and up to PND 7. Subsequently, male pups were distributed equally among dams within the same experimental group to randomize maternal exposure [44]. Animals were ~~sacrificed~~ and processed for assessment of Leydig cell differentiation at 21, 35, and 90 days of age. In addition, primary Leydig cell and Sertoli cell cultures were used in experiments designed to determine that BPA effects result from direct action in testicular cells.

Analysis of Proliferative Activity

Previous studies of Leydig cell development showed that initial steps in Leydig cell differentiation represent a mitotic phase, which increases the number of Leydig cells in the testis. This prepubertal increase in population is critical to attaining and maintaining optimal androgen secretion in the adult testis [20]. For these reasons, PLCs (PND 21) have been routinely used in proliferation assays [23]. Proliferative activity was assessed by [^{3}H] thymidine incorporation using PLCs isolated from male rats at the end of BPA exposure (i.e., in vivo exposure) and BPA-free PLCs incubated in culture media containing BPA (0, 0.01, 10 nM; 18 h) and ovine LH (10 ng/ml; NIDDK), that is, in vitro treatment. Using stereological techniques, Leydig cell numbers were enumerated in testes of control and EDS-treated 60-day-old male rats exposed to BPA in the perinatal period as well as in 90-day-old male rats exposed to BPA without EDS treatment.

Assays of [^{3}H] thymidine incorporation involved incubation of Leydig cells in triplicate in culture media containing 100 ng/ml of LH (NIDDK) and 1 μCi/ml of [^{3}H] thymidine (specific activity, 80 Ci/mmol; lot no. 3106516; DuPont-NEN Life Science Products). After radiolabeling (3 h), Leydig cells were rinsed in Dulbecco PBS containing ethylenediaminetetra-acetic acid (EDTA; catalog no. E-5134; Sigma) and were thereafter divided into $0.3–0.5 \times 10^6$ aliquots and lysed in microcentrifuge tubes containing 0.5 ml of hyamine hydroxide (catalog no. 802387; MP Biomedicals). Cellular [^{3}H] thymidine incorporation was quantified by liquid scintillation counting.

The EDS-treated rat model was used to assess Leydig cell proliferation in vivo. EDS (courtesy of Dr. Earl Gray, Jr., Reproductive Toxicology Branch, U.S. Environmental Protection Agency, Research Triangle Park, NC) was administered to 60-day-old control and BPA-exposed male rats (n = 5) at 80 mg/kg BW in dimethyl sulfoxide/water (1:3, v/v) [23]. The EDS model recapitulates development in vivo because EDS-induced elimination of the original Leydig cell population is followed by new waves of PLCs appearing in the testis [45, 46]. Thus, the EDS model provides data that complement the results of our [^{3}H] thymidine incorporation assays. Following EDS administration, Leydig cell regeneration was allowed until 88 days of age, that is, 28 days post-EDS treatment, when Leydig cell numbers were enumerated using stereological methods. Also, we determined whether BPA induction of proliferative activity early in development affected Leydig cell numbers in the adult testis. Accordingly, the numbers of Leydig cells were enumerated in testes of 90-day-old control and male rats exposed to BPA from GD 12 to PND 21.

Identification of Molecular Targets of BPA Action Affecting Cell Division

In order to determine the mechanisms of BPA action, we performed assays to identify putative molecular targets regulating Leydig cell division. Four different categories were considered: 1) cell cycle proteins (e.g., PCNA, cyclin D3), 2) MAP kinases (e.g., extracellular regulated kinase [MAPK3/1]), 3) hormone transcription factors (e.g., LHCGR, ESR1, AR), and 4) growth factor receptors

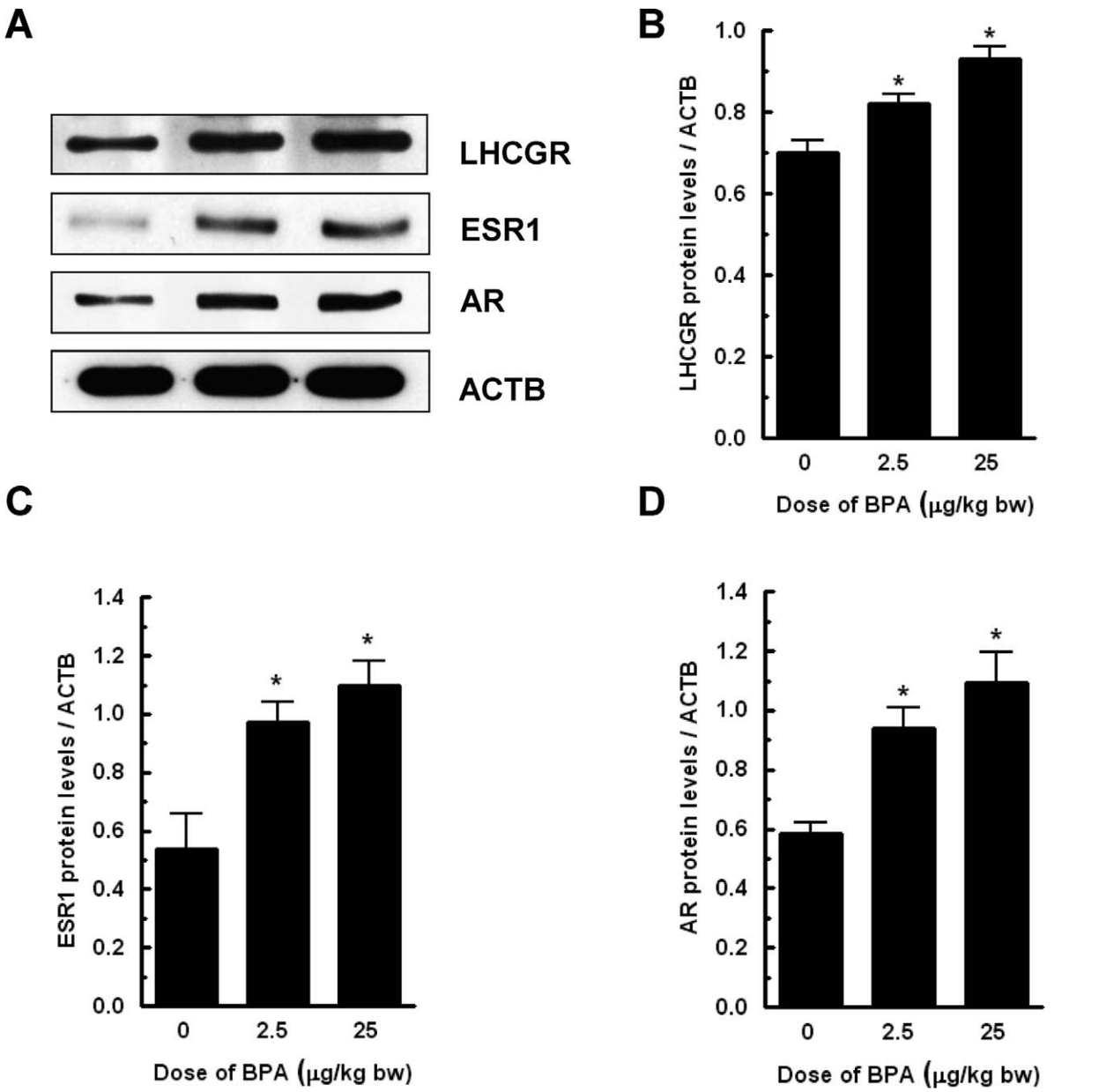

FIG. 3. After perinatal BPA exposure was terminated on Day 21 postpartum, PLCs were isolated and pooled from 28–30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of LH receptor (LHCGR), estrogen receptor 1 (ESR1), and androgen receptor (AR) using specific anti-LHCGR (**A**, **B**), anti-ESR1 (**A**, **C**), and anti-AR (**A**, **D**) antibodies and appropriate secondary antibodies. Western blotting procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). LHCGR, 80 kDa; ESR1, 68 kDa; AR, 110 kDa; ACTB: 42 kDa. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLCs: progenitor Leydig cells at 21 days of age. $*P < 0.05$ versus control.

(e.g., IGF1RB, EGFR). Therefore, PLCs isolated from control and BPA-exposed male and not labeled with [^{3}H] thymidine were processed to obtain whole-cell lysates for Western blot analysis of proteins of interest. IGF1RB and EGFR protein were also measured in BPA-free PLCs after incubation in culture media containing BPA (0, 10 nM) and ovine LH (10 ng/ml) for 18 h. In other experiments, BPA-free PLCs were incubated in culture media containing IGF1 and EGF (10 ng/ml) for 3 h and subsequently processed for [^{3}H] thymidine incorporation and liquid scintillation counting. Furthermore, we isolated Leydig cells from groups of 30, 18, and 8 animals at 21, 35, and 90 days of age, respectively, for analysis of IGF1RB protein levels for comparison to EGFR [47].

Because disruption in secretion of the AMH ligand may contribute to mitogenic effects in Leydig cells, we measured AMHR2 protein levels in Western blots of PLCs at 21 days of age following perinatal BPA exposure. To determine whether expression of the AMHR2 protein correlates directly with proliferative capacity during development, AMHR2 protein levels were analyzed in Western blots of BPA-free Leydig cells obtained at 21, 35, and 90 days of age. Finally, we asked whether BPA acts directly in Sertoli cells to regulate secretion of the AMH ligand using neonatal rat Sertoli cells. This approach is based on our focus on the effects of perinatal (i.e., developmental) exposures and is in agreement with our exposure paradigms in vivo. Neonatal Sertoli cells were isolated from 15 6-day-old male pups not previously exposed to BPA and incubated first in serum-free culture media for 48 h and then in fresh media containing BPA (0, 0.01, and 10 nM) for 24 h. Sertoli cells were harvested at the end of BPA treatment and processed to obtain lysates for measurement of AMH protein by Western blot analysis.

Measurement of Steroidogenic Capacity

Although perinatal exposure of male rats to BPA was previously found to cause a decrease in Leydig cell T production capacity, the sites of BPA-induced

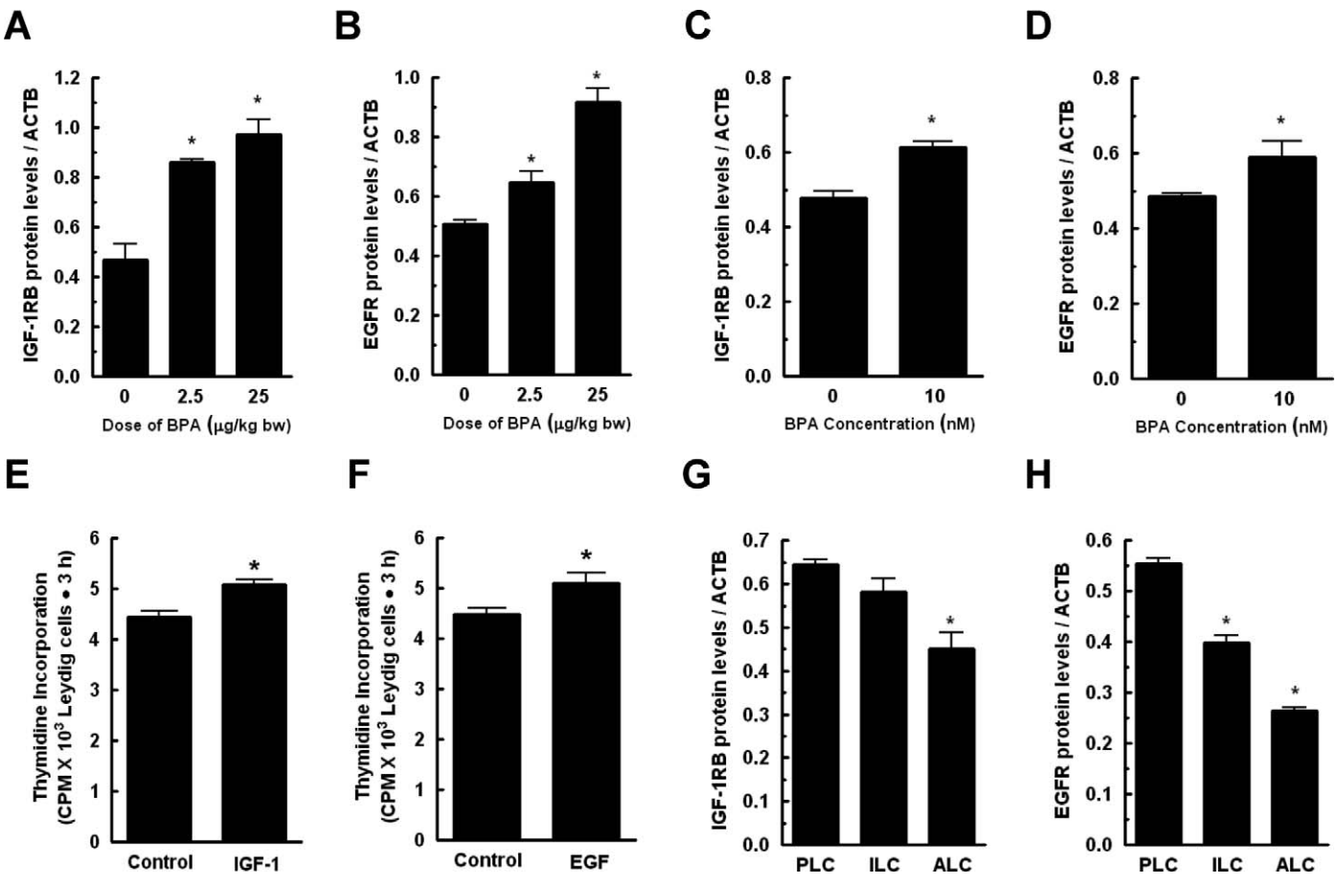

FIG. 4. At termination of perinatal BPA exposure (Day 21 postpartum), PLCs were isolated and pooled from 28–30 male rats per treatment group (**A**, **B**) or were isolated from 35 21-day-old male rats not previously exposed to BPA for incubation in culture media containing BPA and ovine LH (10 ng/mL) for 18 h (**C**, **D, E, F**). IGF1RB and EGFR protein levels were analyzed in Western blots using antisera specific to IGF1RB and EGFR. In addition, BPA-free PLCs were incubated in culture media containing IGF1 or EGF (10 ng/mL) and ovine LH (10 ng/mL) for 3 h and subsequently processed for [^{3}H] thymidine incorporation and scintillation counting (**E**, **F**). Finally, expression of the IGF1RB (**G**) and EGFR protein (**H**) were analyzed in BPA-free PLCs, ILCs and ALCs obtained from 35, 18, and 8 male rats at 21, 35, and 90 days of age. EGFR protein levels were reported previously [47]. Data from in vitro experiments represent results from three separate and independent experiments (**C**, **D**, **E**, **F**). Western blotting procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). IGF1RB, 97kDa; EGFR, 170 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLC, progenitor Leydig cells at 21 days; ILC, immature Leydig cells at 35 days, ALC, adult Leydig cells at 90 days. *$P < 0.05$ versus control.

lesions in the androgen biosynthetic pathway were not identified [19]. Because differentiation is marked by unique biochemical and morphological features at different stages, Leydig cells were isolated and pooled from 21-, 35-, and 90-day-old control and BPA-exposed male rats. This approach allows us to define BPA regulation of steroidogenic capacity in Leydig cells through the entire period of development (neonatal, pubertal, and adult), although BPA exposure was terminated at day 21 postpartum. The amounts of T production were assayed after aliquots of 0.5–2 × 10^5 Leydig cells were incubated in microcentrifuge tubes containing DMEM/F12 culture media and LH (100 ng/ml) for 3 h at 34°C. In addition, we attempted to describe the relationship between Leydig cell T production and peripheral androgen levels by measuring serum T concentrations in animals that were processed to obtain Leydig cells. In all cases, T concentrations were assayed in aliquots of spent media and serum samples by a previously described tritium-based RIA with an interassay variation of 7%–8% [48]. In order to localize BPA-induced lesions, we analyzed the levels of LHCGR, steroidogenic acute regulatory protein (STAR) and steroidogenic enzymes (CYP11A1, HSD3B, CYP17A1, and HSD17B3) in Western blots of ALCs isolated at 90 days of age from control and adult male rats exposed to BPA in the perinatal period (i.e., GD 12–PND 21).

Isolation of Testicular Cells

Leydig cells were isolated from Long-Evans male rats after they were ~~sacrificed~~ by CO_2 asphyxiation and involved collagenase digestion of testis followed by Percoll density centrifugation according to a procedure described previously [49] but excluding the elutriation step. After testis digestion but before Percoll density centrifugation, seminiferous tubules were removed by passage of testicular fractions through nylon mesh (pore size, 0.2 μm; Spectrum Laboratories Inc.). Cell fractions were loaded on to a Percoll gradient for 60 min to isolate bands of Leydig cells. Yields of Leydig cells were estimated with a hemocytometer, whereas purity was assessed by histochemical staining for HSD3B using 0.4 mM etiocholan-3β-ol-17-one enzyme substrate (catalog no. E-5251; Sigma) [50]. In all cases, the culture media consisted of DMEM/F-12 buffered with 14 mM NaHCO3, containing 0.1% BSA and 0.5 mg/ml bovine lipoprotein [51]. PLCs and ILCs were cultured at a density of 1–2.0 × 10^6 cells per well in six-well plates and ALCs at 0.1–0.2 × 10^6 cells per well in 12-well plates (Corning-Costar Company) in an atmosphere containing 5% O_2 and 5% CO_2 and at a temperature of 37°C.

Sertoli cells were isolated according to our usual protocol from 5- or 6-day-old male Long-Evans rats after they were ~~sacrificed~~ by CO_2 asphyxiation [52–~~54]~~–54]. Briefly, testes were decapsulated and subjected to sequential enzymatic digestion using a buffer containing 0.1% collagenase, 0.1% hyaluronidase, and trypsin inhibitor in 0.5% BSA to eliminate peritubular and Leydig cells. The resulting mixed cell suspension was trypsinized to eliminate clumps and resuspended in serum-free DMEM/F-12 media (minimal media) before culture at 37°C in matrigel-coated 12-well plates (Collaborative Research). This method of isolation yields Sertoli cell populations with purity of >95% and preserves the functionality of Sertoli cells for at least 7 days [53, 54]. The media was changed after 4 h to remove spermatogonia followed by culture of Sertoli cells for 48 h prior to treatment with BPA (0, 0.01, 10 nM; 24 h).

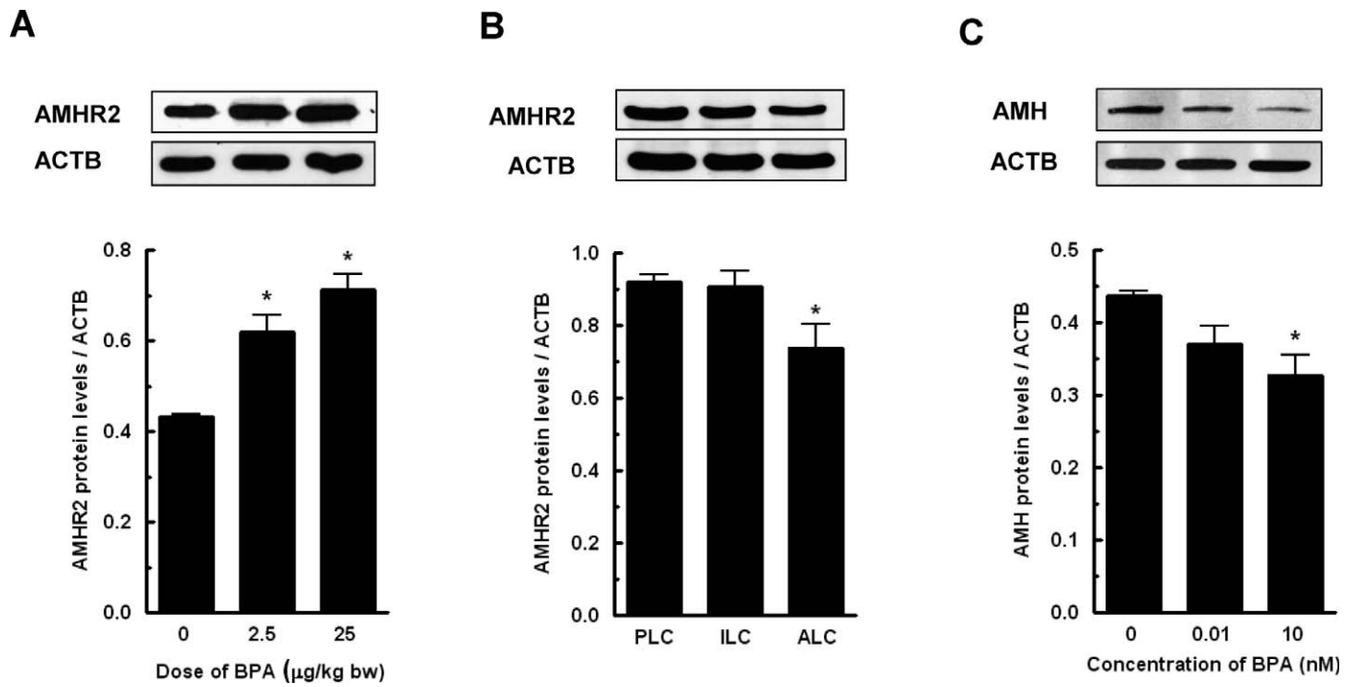

FIG. 5. Following perinatal BPA exposure, which was terminated on Day 21 postpartum, PLCs were isolated and pooled from 28–30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of AMHR2 protein (**A**). Also, developmental expression of the AMHR2 protein was analyzed in BPA-free PLCs, ILCs, and ALCs obtained from 35, 18, and 8 male rats at 21, 35, and 90 days of age (**B**). Finally, AMH protein levels were analyzed in Western blots of neonatal Sertoli cells isolated from 15 6-day-old male rats after incubation in culture media containing BPA for 24 h (**C**). Western blotting procedures were performed using antisera specific to AMHR2 and AMH and appropriate secondary antibodies and were repeated at least four times (**A**, **B**). Protein levels were normalized to β-actin (ACTB). Data from in vitro experiments represent results from three separate and independent experiments (**C**). AMHR2, 63 kDa; AMH, 65 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLC, progenitor Leydig cells at 21 days of age; ILC, immature Leydig cells at 35 days; ALC, adult Leydig cells at 90 days. *$P < 0.05$ versus control.

SDS-PAGE and Western Blot Analysis

Cells were homogenized in T-PER lysis buffer (Pierce Chemical Co.) freshly supplemented with protease inhibitor cocktail (catalog no. 78410; Pierce Biotechnology, Inc.). Tubes were centrifuged at 12000 rpm for 10 min at 4°C to remove cellular debris. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad) using BSA as standard. Protein aliquots of 50-μl whole-cell lysate were dissolved in 50 μl of Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 min at 95°C. Reduced protein lysates were resolved on varying percentages of Tris-HCl acrylamide gels for SDS-PAGE and were transferred to nitrocellulose membranes (catalog no. 1620116; Bio-Rad), which were subsequently incubated with blocking buffer (5% whole milk in 0.1% Tween-20 PBS) for 1 h at room temperature to reduce nonspecific binding by antibody. Membranes were then incubated with primary antibodies in blocking buffer overnight at 4°C (Table 1). On the next day, blots were washed three times in 0.1% tween-20 PBS (TPBS) to remove unbound antibodies before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Membranes were washed four times with 0.1% TPBS and incubated with chemiluminescent developing reagent (catalog no. E2400; Denville Scientific) for 1 min before exposure to X-ray films (catalog no. E-3012; Denville Scientific). The presence of the appropriate proteins was visualized by developing the film and were scanned using an Epson 4180 Perfection scanner (Epson-America). Relative protein amounts in identified immunoblots were measured as optical density of the bands on exposed autoradiographic films using Doc-It LS software (Ultra-Violet Products Ltd). Phosphorylated proteins were normalized to corresponding total or inactive protein levels, whereas other proteins were normalized to β-actin (ACTB).

Stereological Method

Testes from three to five animals per group were analyzed to enumerate Leydig cell numbers. Testes were obtained after whole-body perfusion with 4% paraformaldehyde PBS (pH 7.2) and stored until embedded in paraffin. Sampling of testicular tissue was done according to the fractionator method described previously [23, 55]. Briefly, sections were dewaxed and hydrated before treatment with 0.9% hydrogen peroxide in methanol. Sections were then incubated with 2.5% BSA in humidified chamber, washed in PBS, and incubated overnight with CYP17A1 antibody (sc-46081; Santa Cruz Biotechnology) at room temperature. On the following day, sections were washed and applied with biotinylated anti-goat secondary antibody (BA-5000; Vector Laboratory) for 1 h before washing in PBS. Subsequently, sections were incubated in ABC reagent for 30 min, followed by DAB reagent for 2–5 min. Slides were mounted with VectaShield (Vector) and sealed with clear nail polish. Immunostained sections were digitally captured at 20× by a Nikon Eclipse E600 microscope (Nikon Instruments) equipped with epifluoresence, bright-field, and differential interference optics. Approximately nine or six sections were sampled for three or five fields at 20× magnification from each testis. Images were recorded with a Spot RT Slider digital camera and Spot Advanced software (Diagnostic Instruments). Leydig cell density was determined on the basis of the number of cells present in the defined areas of 6-μM-thick sections. The density of the testis is considered to be approximately 1 mg/ml [56]. Therefore, testis weight was used as an estimate of testis volume (mm^3), and the total number of Leydig cells per testis was determined by multiplying cell density by testis volume.

Statistical Analysis

Data are presented as the mean ± SD. Data describing pregnancy outcome and reproductive parameters up to PND 7 were based on the litter as a unit of measurement, whereas parameters obtained thereafter were collected from randomly selected animals within each treatment group. Leydig cells isolated from control and BPA-exposed animals at 21, 35, and 90 days were pooled from 28–30, 14, and 7–10 animals per group. For developmental studies, Leydig cells were routinely isolated from 35, 18, and 8 animals at 21, 35, and 90 days of age, respectively. When required, culture of isolated Leydig cells were performed in triplicate and subsequently assayed in duplicate or greater in order to facilitate statistical analysis. Experiments performed in vitro were repeated at least three times using Leydig cells isolated from groups of 35

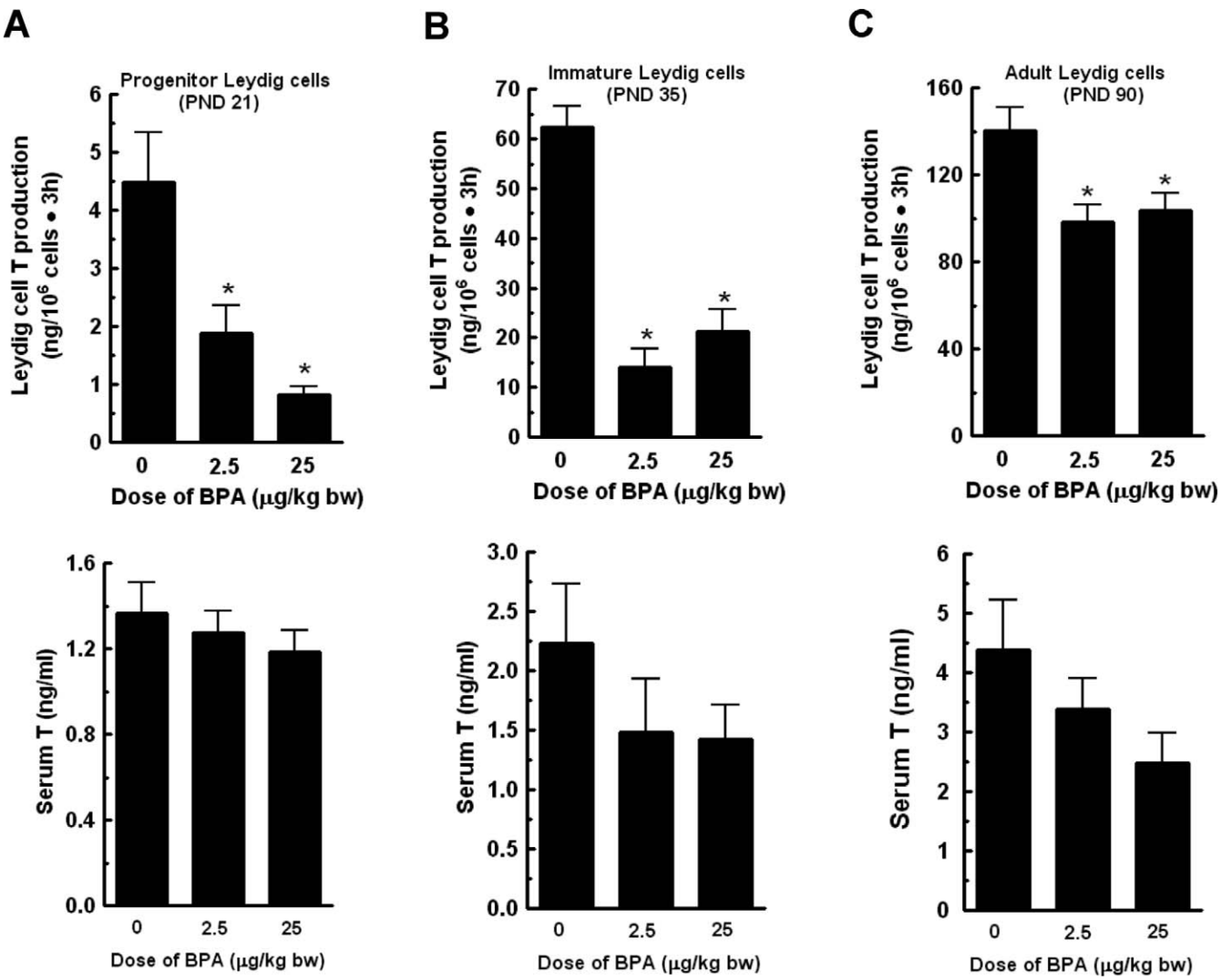

FIG. 6. After perinatal BPA exposure was terminated on Day 21 postpartum, Leydig cells were isolated and pooled from 28–30, 14, and 7–10 male rats per treatment group at 21, 35, and 90 days and were then incubated in culture media in triplicate. Serum was obtained from blood collected from animals at the time of sacrifice. Leydig cell testosterone (T) production and serum T concentrations were analyzed by RIA, which were performed in duplicate. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PND: postnatal day. *$P < 0.05$ versus control.

prepubertal rats at 21 days of age on each occasion. Data were analyzed by unpaired *t*-test for two groups or one-way ANOVA followed by Dunnett's test for multiple group comparisons using GraphPad Prism software. Differences of $P \leq 0.05$ were considered to be significant.

RESULTS

General Observations

Perinatal exposure to BPA did not affect pregnancy outcome, including litter size, birth weights of pups, and pup sex ratio. Body weights, measured at 21, 35, and 90 days of age, were similar in BPA-exposed and control animals ($P > 0.05$). Also, paired and relative testes weights (proportion to body weights) were not affected by BPA. These parameters are shown in Table 2.

BPA Stimulated Proliferative Activity

As determined by [^{3}H] thymidine incorporation, exposure of male rats to BPA by maternal gavage in the perinatal period (i.e., from GD 12 to PND 21) induced proliferative activity in PLCs compared to control (Fig. 1A; $P < 0.05$). The mitogenic effects of BPA result in part from direct BPA action because incubation of PLCs with BPA at 0.01 nM decreased, whereas the 10 nM concentration increased [^{3}H] thymidine incorporation compared to control (Fig. 1B; $P < 0.05$). As expected, EDS administration to 60-day-old control and BPA-exposed male rats eliminated Leydig cells from the testis as indicated by low serum T levels 10 days post-EDS treatment (data not shown). However, 28 days after EDS administration (i.e., PND 88), restoration of the Leydig cell population occurred much faster in the 2.5-μg BPA dose group than in control animals (Fig. 1C; $P < 0.05$). Furthermore, perinatal exposure of male rats to BPA increased Leydig cell numbers at 90 days of age by approximately 16% and 11% in the 2.5- and 25-μg BPA dose groups compared to control (Fig. 1D; $P < 0.05$).

BPA Increased Expression of Mitogenic Regulatory Proteins

Perinatal exposure of male rats to BPA from GD 12 to PND 21 increased PCNA and cyclin D3 protein in PLCs compared

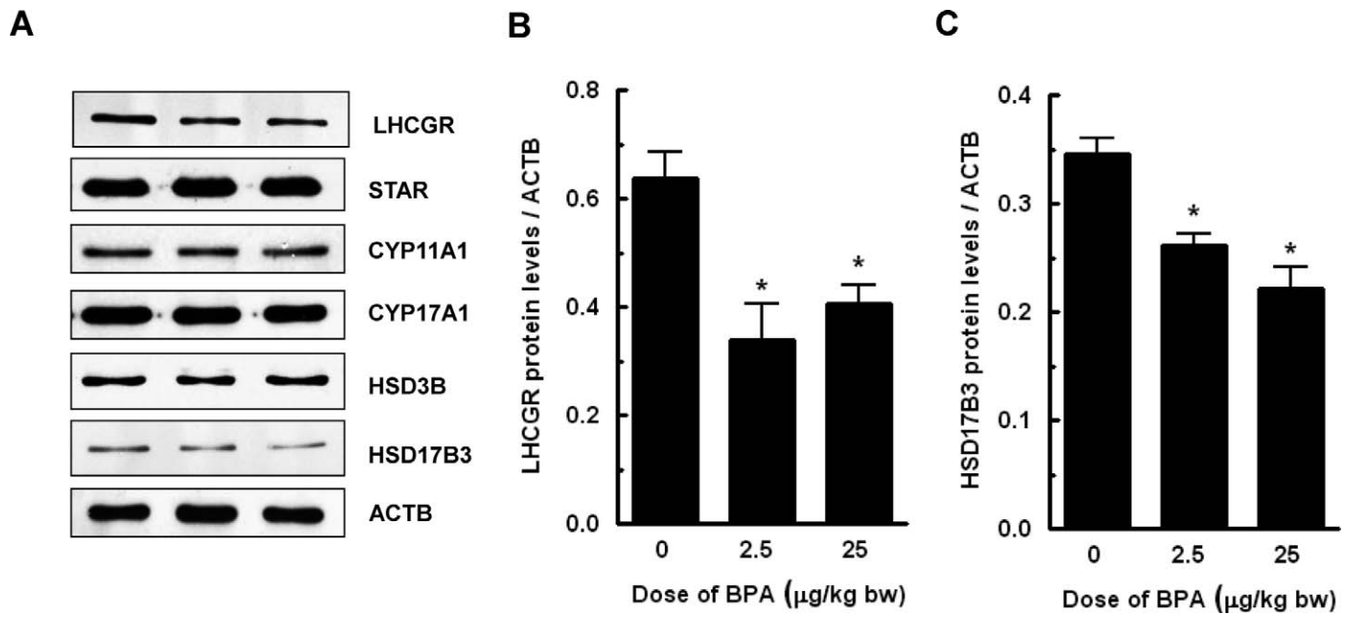

FIG. 7. Adult Leydig cells were isolated and pooled from 7–10 male rats per treatment group at 90 days of age after perinatal exposure to BPA. The androgen biosynthetic pathway was analyzed by Western blotting using anti-LHCGR, anti-STAR, anti-CYP11A1, anti-CYP17A1, anti-HSD3B, anti-HSD17B3 antibodies, and appropriate secondary antibodies. Data represent results from at least four Western blot procedures, and protein levels were normalized to β-actin (ACTB). LHCGR, 85 kDa; STAR, 30 kDa; CYP11A1, 60 kDa; HSD3B, 42 kDa; CYP17A1, 55 kDa; HSD17B3, 35 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. *$P < 0.05$ versus control.

to control (Fig. 2, A and B; $P < 0.05$), which was associated with MAPK3/1 activation as evidenced by increased phosphorylation (p-MAPK3/1; Fig. 2C; $P < 0.05$). In addition, the results of immunoblot analysis showed that perinatal exposure to BPA caused greater levels of the LHCGR (Fig. 3, A and B), ESR1 (Fig. 3, A and C), and AR protein than in control PLCs (Fig. 3, A and D; $P < 0.05$).

Furthermore, exposure of male rats to BPA from GD 12 to PND 21 increased IGF1RB and EGFR protein expression in PLCs compared to control (Fig. 4, A and B; $P < 0.05$) as was also observed in vitro because incubation with BPA (10 nM, 18 h) increased IGF1RB and EGFR protein in PLCs compared to control (Fig. 4, C and D; $P < 0.05$). In addition, incubation with IGF1 and EGF (10 ng/mL, 3 h) also induced proliferative activity in PLCs evidenced by increased [^{3}H] thymidine incorporation compared to control (Fig. 4, E and F; $P < 0.01$). Finally, greater levels of IGF1RB (present study; Fig. 4G) and EGFR protein (Fig. 4H) [47] were measured in PLCs than in ILCs and ALCs ($P < 0.05$).

Expression of the AMHR2 protein in PLCs was increased after exposure to BPA in the perinatal period (i.e., GD 12–PND 21) compared to control (Fig. 5A; $P < 0.05$). There were developmental changes in AMHR2 expression because greater AMHR2 protein was present in highly proliferative PLCs than in ILCs and ALCs (Fig. 5B; $P < 0.05$). In other experiments, BPA treatment (10 nM, 24 h) decreased AMH protein expression in neonatal rat Sertoli cells compared to control (Fig. 5C; $P < 0.05$).

Exposure to BPA Decreased Leydig Cell T Production Associated with Reduced LHCGR and Steroidogenic Enzyme Protein

The results of RIAs showed that Leydig cell T production was decreased at termination of BPA exposure (PND 21) and later in the postnatal period (PND 35 and PND 90; $P < 0.05$). However, decreased Leydig cell T production was not reflected in serum T levels, which were similar in all groups (Fig. 6; $P > 0.05$). In addition, the sites of BPA-induced lesion were identified to be LHCGR and the HSD17B3 enzyme because the levels of LHCGR and HSD17B3 protein were smaller in BPA-exposed ALCs than in control at 90 days (Fig. 7; $P < 0.05$).

DISCUSSION

Although serum BPA levels were not measured in the present study, it was previously determined that exposure of nursing dams to BPA delivered the compound to the offspring at levels that are 300-fold lower; that is, maternal gavage at 100 μg/kg BW represents fetal exposure at 0.32 μg/kg BW [57]. Therefore, maternal exposures to BPA at 2.5 and 25 μg/kg BW represent BPA doses to the offspring of about 8 and 80 pg/kg BW. These dosage regimens reflect BPA levels in the environment [9]. In the present study, gavage of pregnant dams with BPA appeared to not affect pregnancy outcome, including litter size, birth weights of pups, and pup sex ratio as well as body and testes weights in adult animals. A previous study reached a similar conclusion because perinatal exposure of male rats to BPA at 70 μg/kg/day increased body weights at birth compared to control animals, but differences were no longer apparent in adulthood [58]. Therefore, it is likely that low-dose BPA exposure paradigms do not cause sustainable changes in body weight. However, exposure to BPA in the perinatal period, as in this study, affected the two features of Leydig cell differentiation: proliferation and steroid hormone secretion (Fig. 8). Importantly, the results indicated that developmental effects of BPA were present into adulthood. For example, the greater number of Leydig cells in the testis of adult rats at 90 days of age is the result of increased Leydig cell proliferative activity early in development. On the other hand, BPA suppressed development of steroidogenic capacity in Leydig cells, which was evident at the end of the exposure

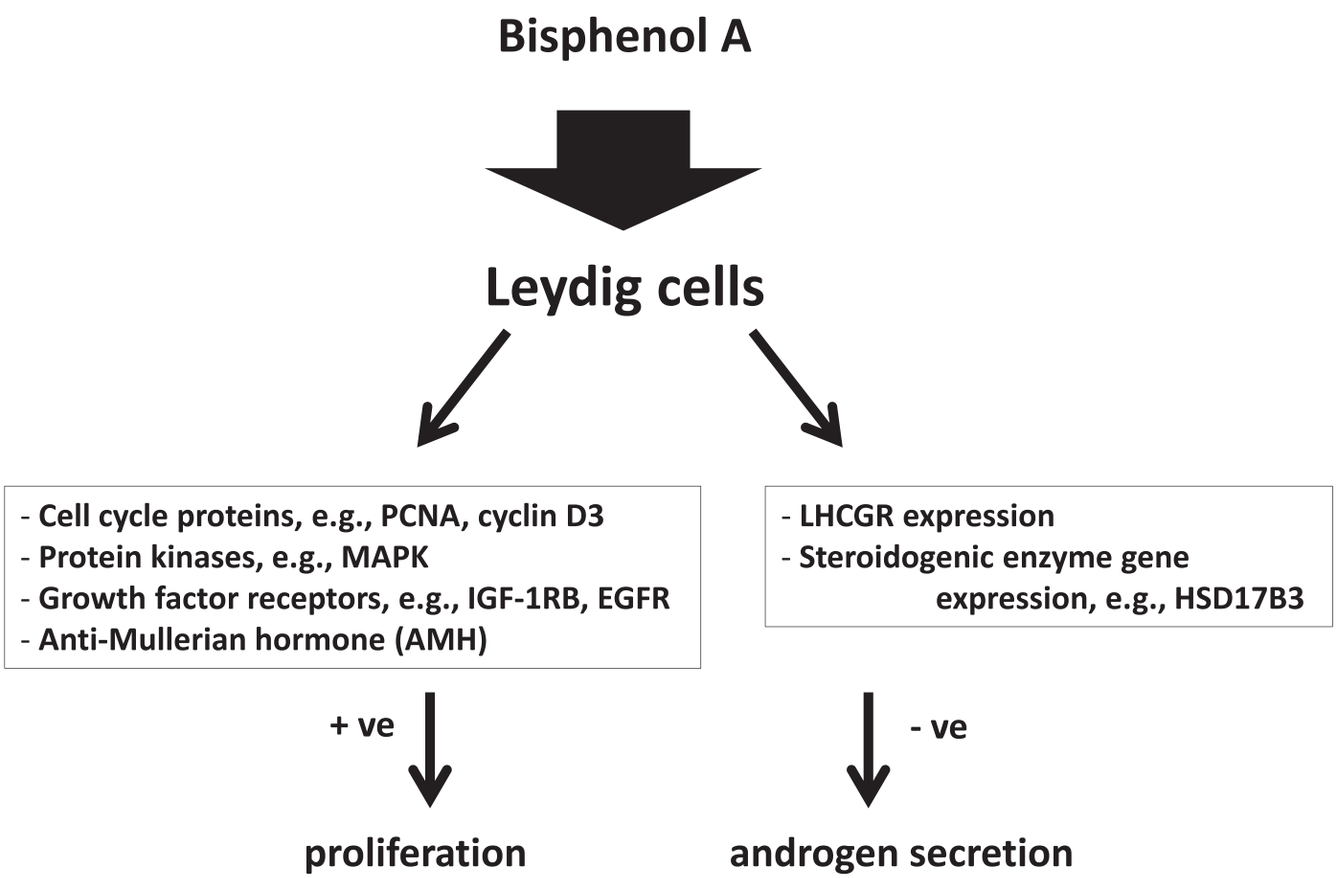

FIG. 8. BPA has the capacity to act directly as a mitogen in Leydig cells by up-regulating expression of cell cycle proteins (e.g., PCNA, cyclin D3). The mitogenic action of BPA is possibly mediated in part by protein kinases (e.g., MAPK3/1), growth factor receptors (IGF1RB, EGFR), and the Sertoli cell-secreted paracrine factor AMH. On the other hand, BPA suppressed protein expression of LH receptors (LHCGR) and the enzyme HSD17B3, thereby decreasing androgen secretion. PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase; IGF1RB, insulin-like growth factor 1 receptor-β; EGFR, epidermal growth factor receptor; AMH, anti-Mullerian hormone; HSD17B3, 17β-hydroxysteroid dehydrogenase type 3.

period (21 days) and into adulthood. Overall, these findings showed that developmental exposures to BPA impact testis development in the male rat.

BPA-induced proliferative activity in Leydig cells was linked to cell cycle progression presumably mediated by PCNA and cyclin D3 [22] and involved MAPK3/1 activation [12, 59, 60]. This is perhaps not surprising because MAPK signaling pathways are known to regulate the actions of growth factors and estrogenic agents in reproductive tract tissues [32, 61], including Leydig cells [24]. Also, BPA increased expression of LHCGR, ESR1, and AR in prepubertal Leydig cells, which has the effect of amplifying hormone-mediated activity supporting tissue differentiation [62, 63]. BPA effects were due, at least in part, to direct action in Leydig cells as confirmed by results of in vitro experiments showing that 10 nM BPA increased, whereas the 0.01-nM concentration decreased [^{3}H] thymidine incorporation. The opposite effects seen at 0.01- and 10-nM concentrations (a 1000-fold difference) are possibly related to the capacity of estrogenic chemicals to cause mostly non-monotonic (nonlinear) effects in estrogen sensitive tissues. The nonmonotonic effect (U shaped or inverted U shaped) is typical of receptor-mediated responses and is thought to vary with molecular events underlying observed changes [64, 65].

The possibility that induction of mitotic activity by BPA involved growth factor receptors was indicated by the presence of greater IGF1RB and EGFR protein levels in BPA-exposed Leydig cells than in control. This interpretation is supported by the results of in vitro assays showing that incubation with IGF1 and EGF increased [^{3}H] thymidine incorporation by Leydig cells. Indeed, IGF1RB- and EGFR-mediated activities were previously determined to increase the rates of Leydig cell mitosis [21, 28, 31], while protein expression of both IGF1RB (this study) and EGFR declined during the transition from prepubertal (PLCs, ILCs) into adult Leydig cells (ALCs) [47]. Specific involvement of IGF1RB and EGFR in mediating BPA effects required their activation, which was not confirmed in the present study. Nevertheless, the results support the view that growth factor receptors are putative mediators of mitogenic signals in Leydig cells.

Moreover, a paracrine relationship is known to exist between Leydig cells and Sertoli cells [34, 35, 66]. Recent reports suggest that Sertoli cell-secreted AMH is a target for endocrine disruptors with estrogenic activity [37]. In this regard, we observed that perinatal exposure of male rats to BPA increased AMHR2 protein in Leydig cells, whereas the results of in vitro assays showed that BPA decreased expression of the AMH ligand in Sertoli cells. Therefore, we propose that deficits in the AMH ligand potentially impact paracrine interactions between Sertoli cells and Leydig cells and likely contribute to the mitogenic effects of BPA in Leydig cells. It is also likely that BPA down-regulation of the AMH protein in Sertoli cells is part of a larger effect on Sertoli cell differentiation. If that were the case, BPA effects in neonatal Sertoli cells may persist into adulthood with potential adverse

effects on sperm production. Therefore, additional studies are required to determine effects on Sertoli cell development and function after BPA exposures occurring during development.

In contrast to stimulatory effects on cell division, perinatal exposure of male rats to BPA suppressed development of steroidogenic capacity in the postnatal period as evidenced by decreased LHCGR and HSD17B3 protein in adult Leydig cells. This finding is relevant because the pituitary gonadotropin LH is the primary factor regulating cholesterol availability and steroidogenic enzyme activity required for androgen biosynthesis. On the other hand, the HSD17B3 enzyme catalyzes the final enzymatic step in androgen biosynthesis, forming T from androstenedione [20]. Although androstenedione accumulation was not measured in the present study, BPA-induced inhibition of androgen secretion likely resulted from diminished LH stimulation and decreased HSD17B3 protein and activity in Leydig cells.

In conclusion, the results of this study demonstrated that perinatal exposure of male rats to BPA impaired postnatal Leydig cell differentiation. Although BPA suppressed T production, serum T levels were unaffected, presumably because of greater Leydig cell numbers, which resulted from increased proliferative activity in the prepubertal period. The present results are similar to reports showing that prenatal exposure of male rats to BPA increased Leydig cell numbers and did not affect serum T levels at 60 days of age [67]. Interestingly, short-term exposure of prepubertal male rats to BPA at 2.4 μg/kg BW from Day 21 to Day 35 postpartum, but not at higher doses, was found to decrease serum T levels [19]. Therefore, it is likely that BPA effects in Leydig cells are affected by the dose, time, and duration of exposure. However, perinatal BPA exposure was found to decrease intratesticular T levels previously [19]. Intratesticular T concentrations are about 30-fold greater (e.g., 70 ng/ml) than serum T levels (e.g., 2 ng/ml) and are not subject to circadian and diurnal fluctuations. Therefore, intratesticular T levels reflect Leydig cell T production capacity better than serum androgen levels in the adult male rat [68]. Although perinatal exposure to BPA decreased intratesticular T by as much as 40% [19], it has been suggested that much less than 50% is required to support sperm production [69]. Because the rat is an efficient sperm producer [70], it is likely that BPA effect of decreasing intratesticular T levels by half will have no impact on quantitative indices of sperm production. However, the present data showed that BPA interferes with AMH secretion by Sertoli cells, which may affect paracrine regulation of testicular cells. Therefore, the long-term effects of BPA on the capacity of somatic cells of the testis (i.e., Sertoli cells) to support qualitative sperm production require further investigation. Interestingly, recent observations have indicated that BPA has the capacity to affect metabolic activity in human adipose tissue and may be a factor in the etiology of metabolic syndromes [71]. Thus, an accumulating body of evidence supports the view that BPA causes biological effects in several body tissues.

REFERENCES

1. Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS, Zoeller RT, Belcher SM. In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 2007; 24:178–198.
2. Pflieger-Bruss S, Schuppe HC, Schill WB. The male reproductive system and its susceptibility to endocrine disrupting chemicals. Andrologia 2004; 36:337–345.
3. Burridge E. Bisphenol A product profile. Eur Chem News 2003; 78: 14–20.
4. Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y. Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 2002; 17:2839–2841.
5. Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 2002; 110:A703–A707.
6. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. Environ Health Perspect 2008; 116:39–44.
7. Lakind JS, Naiman DQ. Bisphenol A (BPA) daily intakes in the United States: estimates from the 2003–2004 NHANES urinary BPA data. J Expo Sci Environ Epidemiol 2008; 18:608–615.
8. Sun Y, Irie M, Kishikawa N, Wada M, Kuroda N, Nakashima K. Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection. Biomed Chromatogr 2004; 18:501–507.
9. Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. Environ Health Perspect 2010; 118:1055–1070.
10. Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 2002; 23:870–881.
11. Matthews JB, Twomey K, Zacharewski TR. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chem Res Toxicol 2001; 14: 149–157.
12. Song KH, Lee K, Choi HS. Endocrine disrupter bisphenol a induces orphan nuclear receptor Nur77 gene expression and steroidogenesis in mouse testicular Leydig cells. Endocrinology 2002; 143:2208–2215.
13. Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci 2003; 75:40–46.
14. Wetherill YB, Petre CE, Monk KR, Puga A, Knudsen KE. The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells. Mol Cancer Ther 2002; 1:515–524.
15. Zoeller RT, Bansal R, Parris C. Bisphenol-A, an environmental contaminant that acts as a thyroid hormone receptor antagonist in vitro, increases serum thyroxine, and alters RC3/neurogranin expression in the developing rat brain. Endocrinology 2005; 146:607–612.
16. Cannon JM, Kostoryz E, Russo KA, Smith RE, Yourtee DM. Bisphenol A and its biomaterial monomer derivatives alteration of in vitro cytochrome P450 metabolism in rat, minipig, and human. Biomacromolecules 2000; 1: 656–664.
17. Hanioka N, Jinno H, Nishimura T, Ando M. Suppression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver. Arch Toxicol 1998; 72:387–394.
18. Kester MH, Bulduk S, van Toor H, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MW, Schuur AG, Brouwer A, Visser TJ. Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J Clin Endocrinol Metab 2002; 87:1142–1150.
19. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology 2004; 145:592–603.
20. Benton L, Shan LX, Hardy MP. Differentiation of adult Leydig cells. J Steroid Biochem Mol Biol 1995; 53:61–68.
21. Ge RS, Hardy MP. Decreased cyclin A2 and increased cyclin G1 levels coincide with loss of proliferative capacity in rat Leydig cells during pubertal development. Endocrinology 1997; 138:3719–3726.
22. Sriraman V, Rao VS, Sairam MR, Rao AJ. Effect of deprival of LH on Leydig cell proliferation: involvement of PCNA, cyclin D3 and IGF-1. Mol Cell Endocrinol 2000; 162:113–120.
23. Sherrill JD, Sparks M, Dennis J, Mansour M, Kemppainen BW, Bartol FF, Morrison EE, Akingbemi BT. Developmental exposures of male rats to soy isoflavones impact Leydig cell differentiation. Biol Reprod 83: 488–501. ?4
24. Yamashita S, Tai P, Charron J, Ko C, Ascoli M. The Leydig cell MEK/ERK pathway is critical for maintaining a functional population of adult Leydig cells and for fertility. Mol Endocrinol 25:1211–1222. ?5
25. Ge RS. Hardy MP. Regulation of Leydig cells during pubertal development. The Leydig Cell in Health and Disease. Contemp Endocrinol 2007; 55–70.
26. Abney TO. The potential roles of estrogens in regulating Leydig cell development and function: a review. Steroids 1999; 64:610–617.

27. Chandrashekar V, Bartke A. Growth factors in Leydig cell function. The Leydig Cell in Health and Disease. Contemp Endocrinol 2007; 263–277.
28. Musa FR, Takenaka I, Konishi R, Tokuda M. Effects of luteinizing hormone, follicle-stimulating hormone, and epidermal growth factor on expression and kinase activity of cyclin-dependent kinase 5 in Leydig TM3 and Sertoli TM4 cell lines. J Androl 2000; 21:392–402.
29. Levine E, Cupp AS, Miyashiro L, Skinner MK. Role of transforming growth factor-alpha and the epidermal growth factor receptor in embryonic rat testis development. Biol Reprod 2000; 62:477–490.
30. Hu GX, Lin H, Chen GR, Chen BB, Lian QQ, Hardy DO, Zirkin BR, Ge RS. Deletion of the Igf1 gene: suppressive effects on adult Leydig cell development. J Androl 31:379–387.
31. Shiraishi K, Ascoli M. Lutropin/choriogonadotropin stimulate the proliferation of primary cultures of rat Leydig cells through a pathway that involves activation of the extracellularly regulated kinase 1/2 cascade. Endocrinology 2007; 148:3214–3225.
32. Smith CL. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol Reprod 1998; 58:627–632.
33. Lee MM, Donahoe PK. Mullerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev 1993; 14:152–164.
34. Lee MM, Seah CC, Masiakos PT, Sottas CM, Preffer FI, Donahoe PK, Maclaughlin DT, Hardy MP. Mullerian-inhibiting substance type II receptor expression and function in purified rat Leydig cells. Endocrinology 1999; 140:2819–2827.
35. Salva A, Hardy MP, Wu XF, Sottas CM, MacLaughlin DT, Donahoe PK, Lee MM. Mullerian-inhibiting substance inhibits rat Leydig cell regeneration after ethylene dimethanesulphonate ablation. Biol Reprod 2004; 70:600–607.
36. Behringer RR, Finegold MJ, Cate RL. Mullerian-inhibiting substance function during mammalian sexual development. Cell 1994; 79:415–425.
37. Chen G, Shinka T, Kinoshita K, Yan HT, Iwamoto T, Nakahori Y. Roles of estrogen receptor alpha (ER alpha) in the regulation of the human Mullerian inhibitory substance (MIS) promoter. J Med Invest 2003; 50: 192–198.
38. Nagai A, Ikeda Y, Aso T, Eto K, Ikeda MA. Exposure of neonatal rats to diethylstilbestrol affects the expression of genes involved in ovarian differentiation. J Med Dent Sci 2003; 50:35–40.
39. Zirkin BR. Spermatogenesis: its regulation by testosterone and FSH. Semin Cell Dev Biol 1998; 9:417–421.
40. Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR, Forsythe DB. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. Lab Anim Sci 1999; 49:530–536.
41. Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Lab Invest 2001; 81:735–747.
42. Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, Thomas S, Thomas BF, Hassold TJ. Bisphenol a exposure causes meiotic aneuploidy in the female mouse. Curr Biol 2003; 13:546–553.
43. Howdeshell KL, Peterman PH, Judy BM, Taylor JA, Orazio CE, Ruhlen RL, Vom Saal FS, Welshons WV. Bisphenol A is released from used polycarbonate animal cages into water at room temperature. Environ Health Perspect 2003; 111:1180–1187.
44. Hilakivi-Clarke L, Cho E, Cabanes A, DeAssis S, Olivo S, Helferich W, Lippman ME, Clarke R. Dietary modulation of pregnancy estrogen levels and breast cancer risk among female rat offspring. Clin Cancer Res 2002; 8:3601–3610.
45. Myers RB, Abney TO. Interstitial cell proliferation in the testis of the ethylene dimethane sulfonate-treated rat. Steroids 1991; 56:91–96.
46. Abney TO, Myers RB. 17 beta-estradiol inhibition of Leydig cell regeneration in the ethane dimethylsulfonate-treated mature rat. J Androl 1991; 12:295–304.
47. Hancock KD, Coleman ES, Tao YX, Morrison EE, Braden TD, Kemppainen BW, Akingbemi BT. Genistein decreases androgen biosynthesis in rat Leydig cells by interference with luteinizing hormone-dependent signaling. Toxicol Lett 2009; 184:169–175.
48. Cochran RC, Schuetz AW, Ewing LL. Age-related changes in conversion of 5 alpha-androstan-17 beta-ol-3-one to 5 alpha-androstane-3 alpha,17 beta-diol and 5 alpha-androstane-3 beta,17 beta-diol by rat testicular cells in vitro. J Reprod Fertil 1979; 57:143–147.
49. Klinefelter GR, Kelce WR, Hardy MP. Isolation and culture of Leydig cells from adult rats. Methods Toxicol 1993; 3A:166–181.
50. Payne AH, Downing JR, Wong KL. Luteinizing hormone receptors and testosterone synthesis in two distinct populations of Leydig cells. Endocrinology 1980; 106:1424–1429.
51. Klinefelter GR, Ewing LL. Optimizing testosterone production by purified adult rat Leydig cells in vitro. In Vitro Cell Dev Biol 1988; 24:545–549.
52. Orth JM. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. Anat Rec 1982; 203:485–492.
53. Karl AF, Griswold MD. Sertoli cells of the testis: preparation of cell cultures and effects of retinoids. Methods Enzymol 1990; 190:71–75.
54. Cooke PS, Zhao YD, Bunick D. Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. Biol Reprod 1994; 51:1000–1005.
55. Petersen PM, Giwercman A, Pakkenberg B. Stereological methods as efficient and unbiased tools to quantitate structures in the testis. Scand J Work Environ Health 1999; 25(suppl 1):31–33.
56. Mori H, Christensen AK. Morphometric analysis of Leydig cells in the normal rat testis. J Cell Biol 1980; 84:340–354.
57. Doerge DR, Vanlandingham M, Twaddle NC, Delclos KB. Lactational transfer of bisphenol A in Sprague-Dawley rats. Toxicol Lett 199: 372–376.
58. Somm E, Schwitzgebel VM, Toulotte A, Cederroth CR, Combescure C, Nef S, Aubert ML, Huppi PS. Perinatal exposure to bisphenol a alters early adipogenesis in the rat. Environ Health Perspect 2009; 117: 1549–1555.
59. Kim JY, Han EH, Kim HG, Oh KN, Kim SK, Lee KY, Jeong HG. Bisphenol A-induced aromatase activation is mediated by cyclooxygenase-2 up-regulation in rat testicular Leydig cells. Toxicol Lett 193: 200–208.
60. Ropero AB, Alonso-Magdalena P, Ripoll C, Fuentes E, Nadal A. Rapid endocrine disruption: environmental estrogen actions triggered outside the nucleus. J Steroid Biochem Mol Biol 2006; 102:163–169.
61. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 2001; 276:36869–36872.
62. Woodham C, Birch L, Prins GS. Neonatal estrogen down-regulates prostatic androgen receptor through a proteosome-mediated protein degradation pathway. Endocrinology 2003; 144:4841–4850.
63. Oliveira CA, Mahecha GA, Carnes K, Prins GS, Saunders PT, Franca LR, Hess RA. Differential hormonal regulation of estrogen receptors ERalpha and ERbeta and androgen receptor expression in rat efferent ductules. Reproduction 2004; 128:73–86.
64. Bouskine A, Nebout M, Mograbi B, Brucker-Davis F, Roger C, Fenichel P. Estrogens promote human testicular germ cell cancer through a membrane-mediated activation of extracellular regulated kinase and protein kinase A. Endocrinology 2008; 149:565–573.
65. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect 2003; 111:994–1006.
66. Rouiller-Fabre V, Carmona S, Merhi RA, Cate R, Habert R, Vigier B. Effect of anti-Mullerian hormone on Sertoli and Leydig cell functions in fetal and immature rats. Endocrinology 1998; 139:1213–1220.
67. Thuillier R, Manku G, Wang Y, Culty M. Changes in MAPK pathway in neonatal and adult testis following fetal estrogen exposure and effects on rat testicular cells. Microsc Res Tech 2009; 72:773–786.
68. Jarow JP, Zirkin BR. The androgen microenvironment of the human testis and hormonal control of spermatogenesis. Ann N Y Acad Sci 2005; 1061: 208–220.
69. Zirkin BR, Santulli R, Awoniyi CA, Ewing LL. Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. Endocrinology 1989; 124: 3043–3049.
70. McLachlan RI, Wreford NG, Meachem SJ, De Kretser DM, Robertson DM. Effects of testosterone on spermatogenic cell populations in the adult rat. Biol Reprod 1994; 51:945–955.
71. Ben-Jonathan N, Hugo ER, Brandebourg TD. Effects of bisphenol A on adipokine release from human adipose tissue: Implications for the metabolic syndrome. Mol Cell Endocrinol 2009; 304:49–54.