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Developmental Exposures of Male Rats to Soy Isoflavones Impact Leydig Cell Differentiation[1]

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ABSTRACT

Testicular Leydig cells, which are the predominant source of the male sex steroid hormone testosterone, express estrogen receptors (ESRs) and are subject to regulation by estrogen. Following ingestion, the two major isoflavones in soybeans, genistin and daidzin, are hydrolyzed by gut microflora to form genistein and daidzein, which have the capacity to bind ESRs and affect gene expression. Thus, the increasing use of soy-based products as nondairy sources of protein has raised concerns about the potential of these products to cause reproductive toxicity. In the present study, perinatal exposure of male rats to isoflavones induced proliferative activity in Leydig cells. Isoflavones have the capacity to act directly as mitogens in Leydig cells, because genistein treatment induced Leydig cell division in vitro. Genistein action regulating Leydig cell division involved ESRs, acting in concert with signaling molecules in the transduction pathway mediated by protein kinase B (AKT) and mitogen-activated protein kinase (MAPK). Enhanced proliferative activity in the prepubertal period increased Leydig cell numbers, which alleviated deficits in androgen biosynthesis and/or augmented serum and testicular testosterone concentrations in adulthood. Together, these observations indicate that the perinatal exposures of male rats to isoflavones affected Leydig cell differentiation, and they imply that including soy products in the diets of neonates has potential implications for testis function.

developmental exposures, genistein, Leydig cells, phytoestrogens, proliferation, soy isoflavones, testicular interstitial cells, testosterone, toxicology

INTRODUCTION

Soy protein and other legumes are consumed in fairly large quantities by populations across the globe [1]. In particular, soy infant formulas serve as dietary sources of nondairy protein and are useful alternatives for infants with cow milk allergy and lactose intolerance [2]. Genistin and daidzin are present at high concentrations (0.2–1.6 mg/g dry wt) in soybeans [3]. Therefore, a significant number of infants in the United States fed soy-based formulas are exposed to genistein and daidzein, which are the biological metabolites of genistin and daidzin [4]. For example, it is estimated that a 4-mo-old infant fed soy formula consumes 28–47 mg of isoflavones per day, or 6–9 mg/kg body weight. Thus, genistein and daidzein may reach micromolar concentrations in infants fed soy protein formulas [5]. Also, serum genistein concentrations as high as 10 μM have been measured in adults following consumption of a phytoestrogen-rich diet [6]. Evidence indicates that genistein and daidzein act as estrogen receptors alpha (ESR1) and beta (ESR2) agonists and/or antagonists and have the capacity to regulate cell proliferation, growth, and function [7]. Because reproductive tract tissues express high levels of steroid hormone receptors, including ESRs, consumption of high levels of soy proteins for prolonged periods of time may affect reproductive tract development and function.

The possibility that feeding of soy protein diets exerts biological effects has been known for a long time. For example, feeding of soy protein supplements to young women for 1 mo was found to affect endocrine profiles and to cause alterations in menstrual cycles [8]. Similarly, premenopausal women fed dietary soy protein for just 14 days exhibited mammary gland tissue proliferation associated with increased expression of the progesterone receptor [9]. The effect of isoflavones on mammary hyperplasia is likely the result of ESR-mediated activity, which upregulates expression of the progesterone receptor in estrogen-sensitive tissues [10]. Also, elderly subjects (age, >60 yr) were more predisposed than younger individuals (age, ~30 yr) to develop symptoms of suppressed thyroid function after ingestion of soybeans for a period of 3 mo [11]. Thus, the biological effects resulting from consumption of soy diets possibly affect all life stages.

In a recent review directed by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction at the National Institute of Environmental Health Sciences, the expert panel concluded that there need be only minimal concern about adverse effects associated with soy-based food products [12]. Regarding male reproduction, this conclusion was based largely on the lack of adverse effects or inconsistencies in reports on reproductive parameters and fertility. For example, a recent multigenerational study linked genistein to mammary hyperplasia in male rats but found no adverse effects on androgen-sensitive end points [13]. However, we observed previously that serum testosterone levels were elevated in groups of rats exposed to dietary isoflavones in the perinatal period [14]. We hereby propose that elevated sex hormone levels in blood may be related to the lack of adverse effects from androgen deficiency after consumption of soy-based diets.

Two Leydig cell generations develop successively in the testis between the periods of embryogenesis and puberty. The

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first generation, designated fetal Leydig cells, differentiates from stromal cells of testis cords starting on Day 12 of gestation in the rat. Once formed, fetal Leydig cells are terminally differentiated, reaching their peak of steroidogenic capacity just before birth on Gestational Day (GD) 19 [15]. On the other hand, the progeny for the second generation, which form adult Leydig cells in the postnatal period, first becomes apparent by Postnatal Day (PND) 11 [16]. These cells are designated progenitor Leydig cells and exhibit intense proliferative capacity. The transition to mature and steroidogenically competent adult Leydig cells involves a dramatic decrease in proliferative capacity as progenitor cells enlarge to form immature Leydig cells at approximately PND 35. Immature cells undergo a final round of cell division to form adult Leydig cells at approximately PND 56. Thus, the most rapid increases in Leydig cell numbers occur between PND 14 and PND 56 in the rat [17]. The decrease in the rate of mitosis accompanying Leydig cell development is accompanied by a gradual increase in the capacity for steroid hormone secretion. Therefore, fully differentiated adult Leydig cells, which constitute the predominant population in the sexually mature testis, lack mitotic activity but have great capacity for steroid hormone secretion.

In mammalian species, Leydig cell development is regulated by the pituitary gonadotropin luteinizing hormone (LH) and by steroid hormones (i.e., androgen and estrogen) [18]. For example, LH is known to stimulate phosphorylation of steroidogenic acute regulatory protein (STAR), which facilitates cholesterol movement into mitochondria for cleavage and enzymatic action. Both LH and steroid hormones are involved in the regulation of enzyme activity, which catalyze the reactions that result in androgen biosynthesis: cytochrome P450 side-chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B), cytochrome $P450_{17\alpha}$-hydroxylase/17–20 lyase (CYP17A1), and 17β-hydroxysteroid dehydrogenase (HSD17B3) [19]. Because the number of Leydig cells in the testis is dependent on proliferative activity that occurs during the prepubertal period [19], modulation of Leydig cell mitosis during the neonatal period of reproductive tract development has the potential to alter Leydig cell numbers in the adult testis. Using immature rat Leydig cells isolated from 35-day-old male rats, we observed previously that genistein acts as a mitogen in Leydig cells [14]. In the present study, we have extended the initial observations in progenitor Leydig cells. To identify the mechanisms by which isoflavones regulate Leydig cell division, we performed proliferation assays in vitro using freshly isolated progenitor cells incubated in culture medium containing genistein. The results show that the mitogenic action of genistein altered Leydig cell numbers and involved ESRs acting in concert with the pathway mediated by protein kinase B (AKT) and mitogen-activated protein kinase (MAPK). Thus, early life exposures of male rats to isoflavones altered Leydig cell development, thereby affecting androgen secretion in the adult testis.

MATERIALS AND METHODS

Experimental Protocol

All animal procedures were approved by the Institutional Animal Care and Use Committee of Auburn University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Time-bred Long-Evans dams (n = 14 per group), weighing approximately 250 g, were allowed 3 days to acclimate in the housing facility of the Department of Laboratory Animal Health, College of Veterinary Medicine, Auburn University. Each dam was housed in a standard plastic cage (length, 0.47 m; width, 0.25 m; height, 0.22 m) lined with wood chip bedding (Lab Products, Inc.), and water was provided in glass bottles. Use of plastic cages and glass bottles was to minimize background exposure of animals to estrogens, as occurs during use of cages made with resins [20]. Animals were maintained under constant conditions of light (12L:12D) and temperature (20–23.3°C), with free access to pelleted food. Diets containing casein (control) and whole soybeans as sources of protein were used in the present study, and diets were formulated to be identical in terms of energy, micronutrients, cholesterol, calcium, and phosphorus. The concentration of isoflavones in experimental diets was 0, 5, 50, or 1000 ppm based on the assayed content of genistin and daidzin and calculation of the equivalent aglycone as specified by the manufacturer (Harlan-Teklad). Pregnant dams were fed diets from GD 12 to PND 21, and all carried the pregnancy to term. Pregnancy outcome, including litter size, pup weight, and male:female ratio of pups, was assessed on PND 1. Although it had been suggested that cross-fostering of pups achieves randomization of the genetic background or its interaction with maternal exposure [21], male rats were reared with their natural littermates and not cross-fostered within groups in the present study. Pregnancy outcome (litter size, pup weight, and sex ratio) and of anogenital distance were measured and analyzed per litter up to PND 5. However, given the small population of Leydig cells in the prepubertal rat testis, each isolation procedure required at least 25 rats from each group. Therefore, male rats were selected randomly from members of each litter per group for analysis of Leydig cell function and other parameters from PND 21.

At weaning (i.e., PND 21), animals were fed the soy-free, casein-based control diet until they were killed. The diets used in the present study were the same formulation as used in our earlier study [14]. Thus, serum levels of isoflavones achieved in the present study are likely similar to levels attained previously, which were in the nanomolar range for free aglycones and reached micromolar amounts for the conjugated moieties [14]. Body and reproductive organ weights (i.e., testis, epididymis, dorsolateral and ventral prostate, and seminal vesicles plus coagulating glands) were obtained from 90-day-old male rats.

Investigation of Proliferative Activity in Leydig Cells

Proliferative activity in Leydig cells was assessed by [^{3}H]thymidine incorporation after in vivo and in vitro exposures to isoflavones. To determine the outcome of altered rates of cell division, Leydig cell numbers in testes of 21-day-old male rats were enumerated using stereological methods. In addition, the ethylene diamine sulfonate (EDS)-administered rat model was used to assess Leydig cell proliferation in vivo. Manufactured by Radian International, EDS (Chemical Abstracts Service no. 462-49-5, lot no. MLE-25448–47, purity 98%) was kindly provided by Dr. Earl Gray, Jr. (Reproductive Toxicology Branch, US Environmental Protection Agency, Research Triangle Park, NC). EDS is a cytotoxin that selectively eliminates Leydig cells from the testis [22]. After EDS administration (80 mg/kg body wt in dimethyl sulfoxide/water [1:3, v/v]), Leydig cell repopulation of the testis interstitium reflects proliferative activity, correlates with testosterone secretion, and is affected by agents with estrogenic activity [23–25]. Therefore, we administered EDS to 60-day-old male rats and measured serum testosterone concentrations as a marker for Leydig cell numbers. For in vitro experiments, aliquots of Leydig cells, isolated from a separate group of rats not previously exposed to isoflavones, were incubated in triplicate with genistein (0, 0.001, 0.1, and 10 μM; catalog no. G-103, lot no. 0103070; Indofine Chemical Company) in Dulbecco modified Eagle medium/Ham F-12 culture medium containing ovine LH for 3 h (10 ng/ml; National Institute of Diabetes, Digestive, and Kidney Diseases). The concentrations of genistein were chosen to approximate the range of aglycone or free genistein concentrations present in blood after consumption of soy-based diets [5, 6].

Several hormones, including the pituitary gonadotropin LH, and androgen receptor (AR) and ESR agonists are known to modulate expression of cognate receptors, thereby modulating LH and steroid hormone-stimulated tissue function [26, 27]. Because proliferative activity is the primary feature of progenitor Leydig cells and is subject to hormonal regulation [28], we analyzed LH receptor (LHR), AR, and ESR1 protein expression levels in Leydig cells isolated from 21-day-old male rats exposed to isoflavones in the perinatal period.

Proliferation assays. Leydig cells from two sources were used in proliferation assays: 1) Leydig cells isolated from 21-day-old male rats exposed to isoflavones in the maternal diet, and 2) freshly isolated Leydig cells treated with genistein in vitro. In either case, aliquots of Leydig cells were incubated in triplicate in culture medium containing 10 ng/ml of LH and 1 μCi/ml of [^{3}H]thymidine for labeling (specific activity, 80 Ci/mmol; lot no. 3106516; DuPont-NEN Life Science Products). After labeling (3 h), Leydig cells were rinsed in Dulbecco PBS containing ethylenediaminetetra-acetic acid (EDTA; catalog no. E-5134, lot no. 074K0004; Sigma) and were divided into

aliquots of 0.3–0.5 × 10^6 cells and lysed in microcentrifuge tubes containing 0.5 ml of hyamine hydroxide (catalog no. 802387, lot no. 8493J; MP Biomedicals). Cellular [^{3}H]thymidine uptake was quantified by liquid scintillation counting. In addition, unlabeled control and genistein-treated Leydig cells were harvested and counted to determine cell numbers. To determine whether proliferation was associated with cell-cycle progression, Leydig cells were processed to obtain whole-cell lysates to measure levels of proliferating cell nuclear antigen (PCNA), cyclin D3, and pRB^{Ser780}. Activation of RB is caused by cyclin-CDK complexes and results in the release of free E2F transcription factors and activation of genes required for DNA synthesis, which are required for cell-cycle progression [29]. Measurement of cyclin D3 and pRB^{Ser780} levels helped us to validate that increased Leydig cell numbers resulted primarily from cell-cycle progression and mitosis.

AKT and genistein action. Evidence indicates that AKT acts as a cell survival factor and increases the cell population in several tissues [30]. Therefore, experiments were performed to determine whether genistein-induced Leydig cell proliferation involves AKT. After exposure to isoflavones in vivo or genistein treatment in vitro (0.1 μM, 3 h), Leydig cells were processed to obtain whole-cell lysates, which were analyzed for AKT activation (i.e., phosphorylation on serine residue 473 [pAKT^{Ser473}]). In separate experiments, aliquots of Leydig cells were incubated for 1 h in culture medium containing a specific AKT kinase inhibitor (1L6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate; catalog no. 124005, lot no. D00031576, 0.1 μM; Calbiochem) and then in fresh medium containing genistein (0.1 μM, 3 h). Leydig cells were then processed to measure cellular [^{3}H]thymidine uptake. The concentration of kinase inhibitor was determined in pilot experiments not to affect Leydig cell proliferation when acting alone. In experiments designed to determine whether AKT is differentially expressed during development, pAKT^{Ser473} levels were analyzed in Leydig cells isolated from 21-, 35-, and 90-day-old male rats not previously exposed to isoflavones.

Investigation of estrogenic activity and Leydig cell division. Regulation of cell proliferation by ESR-mediated activity was assessed by [^{3}H]thymidine uptake and cell numbers after incubation of Leydig cells in culture medium containing 10 ng/ml of LH and 17β-estradiol (E_2; 10 nM, 3 h). Also, control untreated and E_2-treated Leydig cells were processed to obtain whole-cell lysates for Western blot analysis of PCNA protein expression. Because E_2 stimulated Leydig cell proliferation, experiments were performed to determine whether genistein action was related to ESR-mediated activity. Aliquots of progenitor Leydig cells were incubated in culture medium with and without the pure antiestrogen ICI 182,780 (0.1 μM, 1 h) and then in fresh medium containing genistein (0.1 μM, 3 h). The ICI 182,780 compound (catalog no. 1047, lot no. 18A/63825; Tocris Chemicals, Inc.) has the capacity to prevent ligand binding of ESR1 and ESR2 [31, 32]. The concentration of the antiestrogen was determined in pilot experiments not to affect Leydig cell proliferation when acting alone. After treatment, Leydig cells were assessed for [^{3}H]thymidine uptake and cell numbers.

Because MAPKs are known to mediate several pathways regulating cellular function and proliferation [33], experiments were performed to determine whether MAPK-mediated pathways are involved in genistein regulation of Leydig cell division. Leydig cells isolated from male rats exposed to dietary isoflavones were processed to analyze activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2 or MAPK3 and MAPK1). In addition, we analyzed the pattern of MAPK3/1 activation in Leydig cells freshly isolated from 21-, 35-, and 90-day-old male rats. Finally, we attempted to validate results from analysis of MAPK activation by incubating Leydig cells in medium with and without the specific MAPK inhibitor PD 98095 (0.1 μM, 1 h; catalog no. 513000, lot no. B73477; Calbiochem) and then in fresh medium containing genistein (0.1 μM, 3 h). The concentration of PD 98095 was determined in pilot experiments not to affect Leydig cell proliferation when acting alone. After treatment, Leydig cells were analyzed for cellular [^{3}H]thymidine uptake. To investigate the possibility of cross-talk between ESR-, AKT-, and MAPK-mediated pathways, activation of MAPK3/1 was investigated in genistein-treated Leydig cells (0.1 μM, 3 h) after pharmacological inhibition of the AKT kinase and blockade of the ESR to prevent binding by genistein. Also, AKT activation was analyzed in genistein-treated Leydig cells, which were pretreated with the ICI 182,780 compound.

Assessment of Steroidogenic Capacity in Fully Differentiated Adult Leydig Cells

Leydig cells were isolated from 90-day-old rats exposed to soy isoflavones in the perinatal period (GD 12 to PND 21). To measure testosterone production capacity, Leydig cells were incubated in culture medium for 3 h at 34°C, and testosterone concentrations were analyzed in aliquots of spent media by radioimmunoassay. The androgen biosynthetic pathway was evaluated by immunoblot analysis of STAR protein and steroidogenic enzymes involved in androgen biosynthesis (CYP11A1, HSD3B, CYP17A1, and HSD17B3).

Procedure for isolation of Leydig cells. Animals were killed by CO_2 asphyxiation. Isolation of Leydig cells involved collagenase digestion of testis followed by Percoll density centrifugation according to a procedure described previously [34] 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.). Filtration of cell fractions was omitted if Leydig cells were isolated from adult rats (age, 60–90 days) when seminiferous tubules were sedimented by gravity in 2 mg/ml of bovine serum albumin solution. Cell fractions were loaded on to a Percoll gradient for 60 min to isolate bands of Leydig cells. Leydig cell numbers 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, lot no. 11K4058; Sigma) [35].

Immunohistochemistry and stereology. Testes were obtained after whole-body perfusion of rats with 4% paraformaldehyde (catalog no. 19200, lot no. 090820; Electron Microscopy Sciences) and stored until embedded in paraffin. Testes from four animals per group were analyzed in this procedure. To enumerate Leydig cell numbers, sampling of testicular tissue was done according to the fractionator technique [36]. In every fractionation step, the selection of a set of blocks or 6-μm-thick sections was performed with systematic random sampling. Identification of Leydig cells was facilitated by processing tissue for immunocytochemistry and staining with a polyclonal antibody specific for the *Cyp17a1* enzyme (sc-46081; Santa Cruz Biotechnology). Sections were examined using a Nikon Eclipse E600 microscope (Nikon Instruments) equipped with epifluoresence, bright-field, and differential interference optics. Images were made with a Spot RT Slider digital camera and Spot Advanced software (Diagnostic Instruments). Leydig cell density was determined based on the number of cells present in the defined area of the 6-μm-thick section. The density of the testis is considered to be approximately 1 mg/ml [37]. 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 the cell density by the testis volume.

SDS-PAGE and Western blot analysis. Leydig cells were homogenized in lysis buffer containing a protease inhibitor cocktail, including 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 0.5 M EDTA, 2 mM leupeptin, and 1 mM peptatin (catalog no. 78410, lot no. KE 121678; Pierce Biotechnology, Inc.). Tubes were centrifuged at 15 000 × *g* for 15 min at 4°C to remove cellular debris. Protein concentration was measured using the Biorad protein assay (Bio-Rad), with bovine serum albumin as standard. Protein aliquots (10–20 μg) in Laemmli sample buffer were resolved on 10% Tris-HCl mini acrylamide gels for SDS-PAGE and electrotransferred to nitrocellulose membranes (Bio-Rad), which were subsequently incubated with primary antibodies recognizing PCNA (mouse monoclonal, sc-53409), cyclin D3 (rabbit polyclonal, sc-182), total AKT (rabbit polyclonal, sc-8312), RB (mouse monoclonal, sc-102), pRB^{Ser780} (goat polyclonal, sc-12901) phosphorylated AKT (pAKT^{Ser473}; rabbit polyclonal, sc-7985), LHR (rabbit polyclonal, sc-25828), AR (rabbit polyclonal, sc-815), ESR1 (mouse monoclonal, ab 2746–50), STAR (rabbit polyclonal), CYP11A1 (goat polyclonal, sc-18043), HSD3B (rabbit polyclonal, sc-28206), CYP17A1(goat polyclonal, sc-46081), HSD17B3 (goat polyclonal, sc-66415), and β-actin or ACTB (goat polyclonal, sc-1616). Antibodies were obtained from Santa Cruz Biotechnology except for STAR (courtesy of Dr. Doug Stocco, Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX) and ESR1 (Abcam, Inc.). Blots were washed to remove unbound antibodies before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Membranes were incubated with chemiluminescent developing reagent (catalog no. E2400; Denville) for 1 min and subsequently exposed to x-ray film (catalog no. E-3012; Denville). The presence of the appropriate proteins was visualized by developing the film, and levels of expression were quantified using the Epson 4180 Perfection scanning software (Epson-America). Relative protein amounts in identified immunoblots were measured as optical density of the bands on exposed Autorad films using Doc-lt 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.

Statistical analysis. Data are presented as the mean ± SD. Data describing pregnancy outcome and reproductive parameters up to PND 5 were based on the litter as a unit of measurement, whereas parameters obtained after PND 21 were collected from randomly selected animals within each diet group. Assays involving material collected from animal studies were performed three to five times. Experiments performed in vitro were repeated at least four times except for those investigating cross-talk between signaling pathways, which were performed twice. Data were analyzed by one-way ANOVA followed by Dunnett test for multiple group comparisons (GraphPad, Inc.). Experiments involving only two groups were analyzed by the unpaired *t*-test for comparison. Differences of $P \leq 0.05$ were considered to be significant.

TABLE 1. Pregnancy outcome and reproductive parameters in male rats.

Parameter	Isoflavone levels in maternal diet (ppm)			
	0	5	50	1000
Litter size (number of pups)[a]	13.2 ± 0.8	12.2 ± 0.6	12.6 ± 0.5	13.1 ± 0.2
Pup sex ratio (male:female)[a]	1.5 ± 0.3	1.1 ± 0.2	1.8 ± 0.4	1.2 ± 0.2
Body weights (g, PND 1)[a]	6 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6 ± 0.1
Anogenital distance (mm, PND 5)[a]	7.29 ± 0.25[c]	7.95 ± 0.3[c]	8.74 ± 0.3[d]	7.46 ± 0.33[c]
Body weights (g, PND 21)[b]	64.2 ± 0.8	62.1 ± 1.0	64.3 ± 1.3	61.9 ± 0.9
Paired testis weights (g, PND 21)[b]	0.29 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.29 ± 0.01
Body weights (g, PND 90)[b]	510 ± 11	538 ± 17	529 ± 10	522 ± 15
Paired testis weights (g, PND 90)[b]	3.5 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.8 ± 0.1
Paired epididymal weight (g, PND 90)[b]	1.12 ± 0.1	1.23 ± 0.03	1.16 ± 0.04	1.19 ± 0.07
Dorsolateral prostate (g, PND 90)[b]	0.63 ± 0.06	0.55 ± 0.01	0.59 ± 0.04	0.61 ± 0.04
Ventral prostate (g, PND 90)[b]	0.71 ± 0.04	0.69 ± 0.04	0.74 ± 0.06	0.68 ± 0.03
Seminal vesicles (g, PND 90)[b]	0.81 ± 0.06	0.77 ± 0.06	0.86 ± 0.03	0.85 ± 0.04

[a] Values were based on the litter as a unit of measurement with n = 14.
[b] Values were based on measurements from randomly chosen male rats from each group with n = 10–12.
[c,d] Anogenital values with different superscripts are significantly different ($P < 0.05$).

RESULTS

Pregnancy outcome (e.g., litter size, birth weight of pups, pup sex ratio), anogenital distance in neonatal rats, as well as accessory sex organ and testes weights in adult rats are shown in Table 1. The anogenital distance was greater in the 5-ppm diet group and was significantly larger in the 50-ppm diet group ($P < 0.05$).

Perinatal Exposure to Isoflavones Induced Proliferative Activity and Increased LHR, AR, and ESR1 Protein Expression in Progenitor Leydig Cells

Exposure of male rats to soy isoflavones induced proliferative activity in progenitor Leydig cells as determined by [^{3}H]thymidine uptake (Fig. 1A). Increased cell proliferation was related to greater PCNA and cyclin D3 protein expression (Fig. 1, B–D). Also, exposure to the 5- or 50-ppm diet increased LHR and ESR1 protein expression in Leydig cells compared to control (Fig. 2, A–D) ($P < 0.05$), whereas AR levels were increased only in the 5-ppm diet group ($P < 0.05$). Furthermore, serum E_2 levels were greater in prepubertal male rats (PND 21) exposed to soy isoflavones than in control animals (Fig. 2E).

Incubation in culture medium containing genistein (3 h) induced Leydig cell proliferation, as indicated by increased [^{3}H]thymidine uptake at 0.1 and 10 μM (Fig. 3A) ($P < 0.05$). Further evidence of proliferative activity was provided by increased numbers of Leydig cells in genistein-treated versus control untreated cultures (Fig. 3B). Western blot analysis demonstrated that genistein treatment was related to increased PCNA protein expression and phosphorylation of the RB protein on serine residue 780 (pRB^{Ser780}), as shown in Figure 3, C and D ($P < 0.05$).

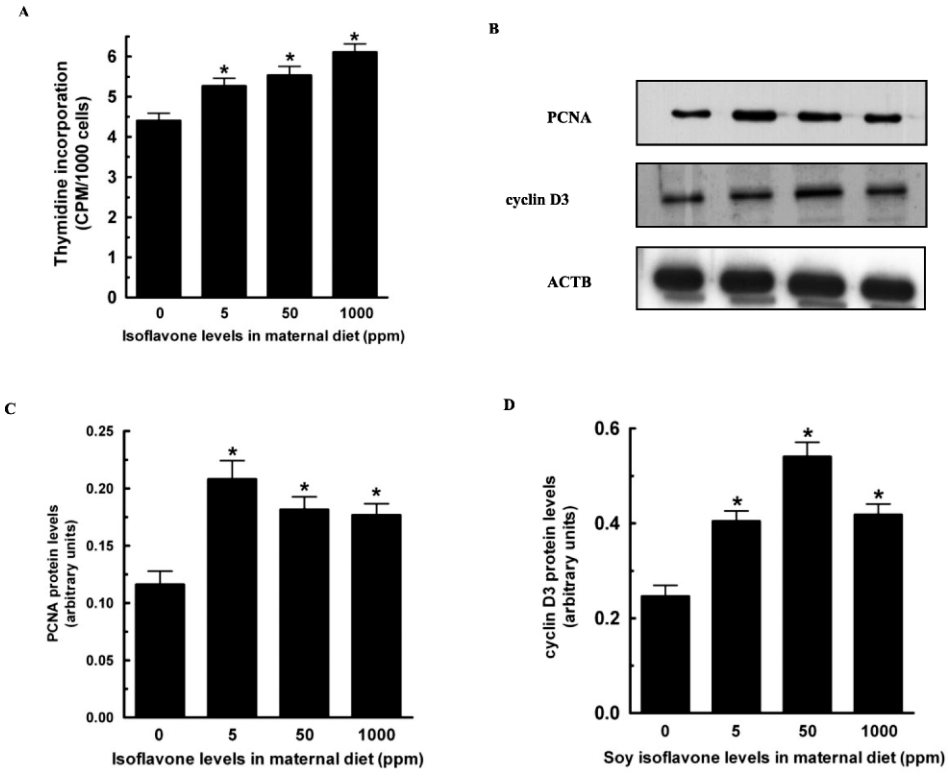

FIG. 1. Proliferative activity was determined by [^{3}H]thymidine uptake (**A**) by progenitor Leydig cells after exposure of male rats to dietary isoflavones. Expression of cell-cycle proteins (PCNA [**B** and **C**] and cyclin D3 [**B** and **D**]) were analyzed by Western blotting and normalized to β-actin (ACTB). Data represent results from densitometric analysis of at least four Western blots. PCNA = 36 kDa, cyclin D3 = 35 kDa. *$P < 0.05$ versus control.

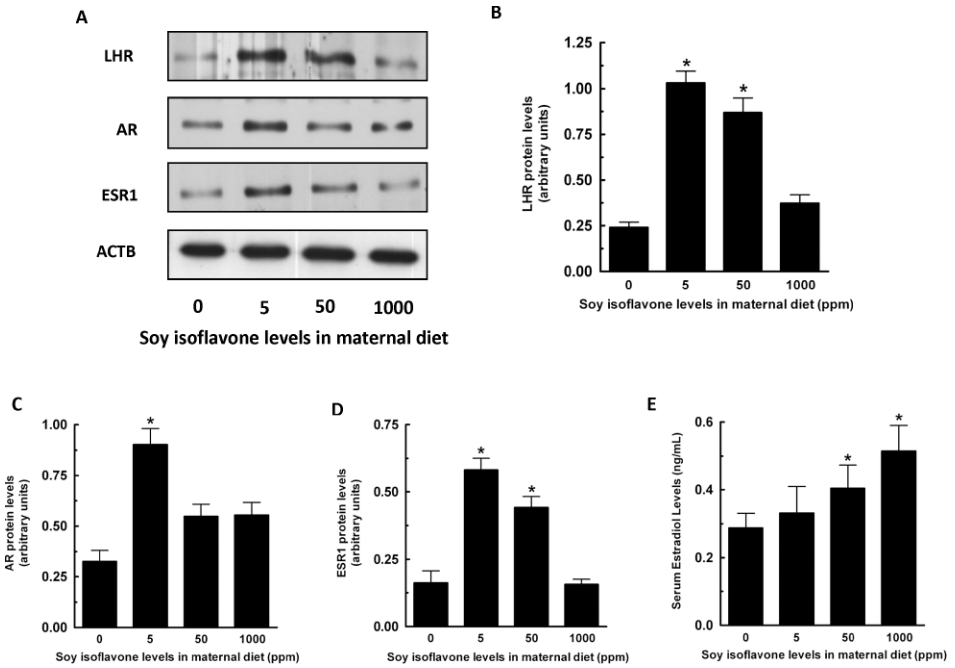

FIG. 2. Expression of LHR, AR, and ESR1 protein was analyzed in progenitor Leydig cells isolated from male rats exposed to dietary isoflavones. Specific anti-LHR (**A** and **B**), anti-AR (**A** and **C**), and anti-ESR1 (**A** and **D**) antibodies and appropriate secondary antibodies were used in Western blotting procedures, which were repeated four to five times. Protein levels were normalized to β-actin (ACTB). Serum was separated from blood and analyzed for E_2 concentrations as assayed in duplicate by radioimmunoassay (**E**) (n = 15). LHR = 80 kDa, AR = 110 kDa, ESR1 = 60 kDa. *$P <$ 0.05 versus control.

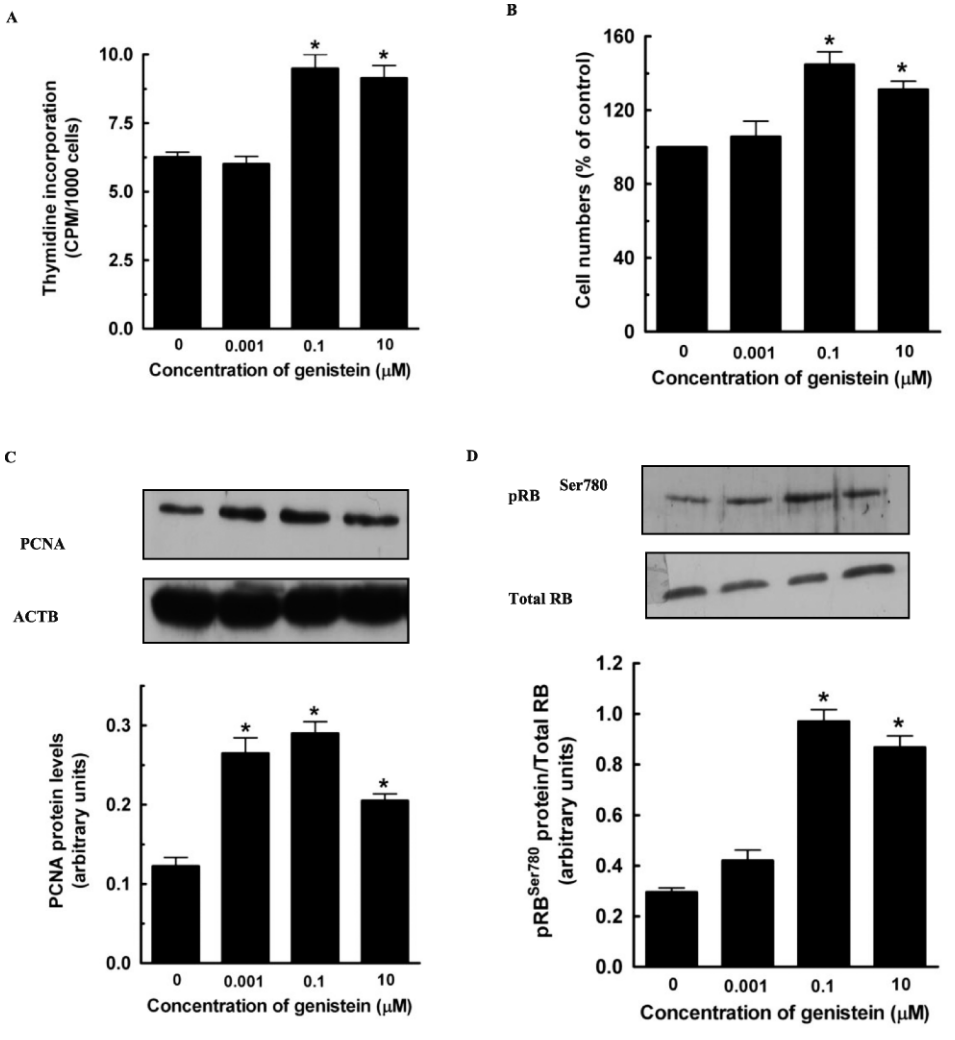

FIG. 3. Proliferative activity in genistein-treated Leydig cells was determined by cellular [^{3}H]thymidine uptake (**A**) and changes in cell numbers (**B**). Expression levels for PCNA (**C**) and pRB^{Ser780} were analyzed by Western blotting (**D**). Immunoblots are representative of four to five Western blotting procedures from three separate and independent experiments. PCNA levels were normalized to β-actin (ACTB), whereas pRB^{Ser780} was normalized to total (inactive) RB levels. PCNA = 36 kDa, RB and pRB^{Ser780} = 110 kDa, ACTB = 42 kDa. *$P <$ 0.05 versus control.

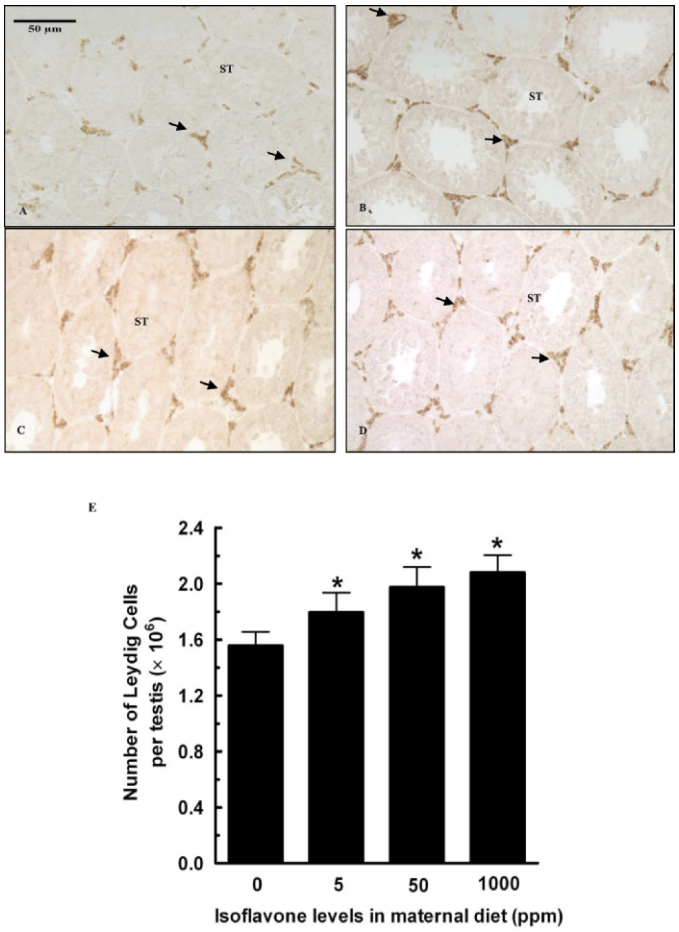

FIG. 4. Leydig cells were localized to testes of 21-day-old male rats exposed to dietary isoflavones (**A**) and enumerated by stereological methods (**E**). Immunolocalization of Leydig cells was achieved using CYP17A1 (Santa Cruz Biotechnology), a biotinylated secondary antibody, and visualization with diaminobenzidine as substrate (Vector Laboratories). Immunoreactivity was observed only in Leydig cells (arrows) localized to interstitial tissue between seminiferous tubules (ST). Photomicrograph is representative of testis interstitium from control animals (**A**) and male rats exposed to 5 ppm (**B**), 50 ppm (**C**), or 1000 ppm (**D**) of isoflavones in maternal diet from GD 12 to PND 21. All sections are at the same magnification. Bar = 50 μm.

Leydig Cell Numbers Are Increased in Testes of Male Rats Exposed to Soy Isoflavones in the Perinatal Period

After immunohistochemical localization, greater Leydig cell numbers were apparent in testes of male rats exposed to soy isoflavones compared to control (Fig. 4, A–D). Indeed, the results of stereological analysis showed that compared to control, Leydig cell numbers were increased by approximately 35%, 40%, and 50% in the 5-, 50-, and 1000-ppm diet group, respectively (Fig. 4E) ($P < 0.05$). The presence of a greater population of Leydig cells in isoflavone-exposed animals was further indicated by increased testicular testosterone concentrations at 35 days of age in the 1000-ppm diet group, although steroidogenic capacity was decreased in Leydig cells (Fig. 5) ($P < 0.05$). Whereas serum testosterone levels were decreased 7 days after EDS administration (Fig. 6A), the levels were restored much faster in isoflavone-exposed animals compared to control animals 28 days after EDS administration (Fig. 6B) ($P < 0.05$). The effect of enhanced Leydig cell proliferation in the prepubertal period was manifested in greater numbers of Leydig cells recovered from testes of isoflavone-exposed rats versus control animals at 90 days of age (Fig. 6C).

Genistein Regulation of Leydig Cell Division Is Mediated in Part by AKT

Exposure of male rats to isoflavones caused greater levels of $pAKT^{Ser473}$ in Leydig cells (Fig. 7A). Similarly, genistein treatment of Leydig cells caused activation of AKT in vitro (Fig. 7B), which was abrogated by pharmacological inhibition of the AKT kinase (Fig. 7C). A role for AKT in Leydig cell division was suggested by measurement of greater levels of $pAKT^{Ser473}$ in progenitor Leydig cells (PND 21) and immature Leydig cells (PND 35) than in fully differentiated adult Leydig cells (PND 90) (Fig. 7D). Together, these observations imply involvement of the AKT-mediated signaling pathway in genistein regulation of Leydig cell division.

Mitogenic Action of Genistein Is Associated with Estrogenic Activity and Is Mediated in Part by the MAPK Family

Incubation of Leydig cells with E_2 increased cellular [^{3}H]thymidine uptake (Fig. 8A) and cell numbers (Fig. 8B) and was related to greater PCNA protein expression (Fig. 8C) ($P < 0.05$). The mitogenic action of genistein was also linked to estrogenic activity, because pharmacological inhibition of ESRs by pretreatment with the antiestrogen ICI 182,780 abrogated the mitogenic action of genistein in Leydig cells

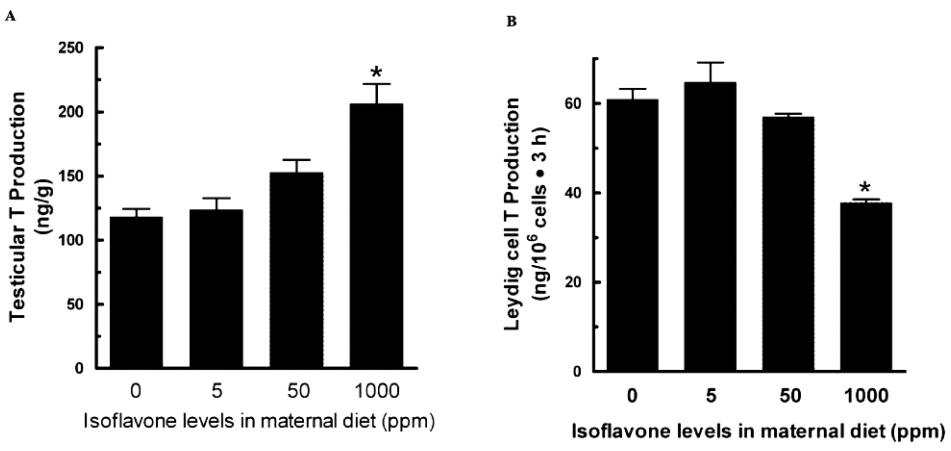

FIG. 5. Testosterone (T) production by testicular explants (**A**) and Leydig cells (**B**) collected from testis of 35-day-old male rats exposed to dietary isoflavones. Testicular explants from 10 to 12 animals and Leydig cells pooled from 15 to 18 animals per group were incubated in triplicate in culture medium for 3 h. The levels of testosterone production were assayed in duplicate by radioimmunoassay. *$P < 0.05$ versus control.

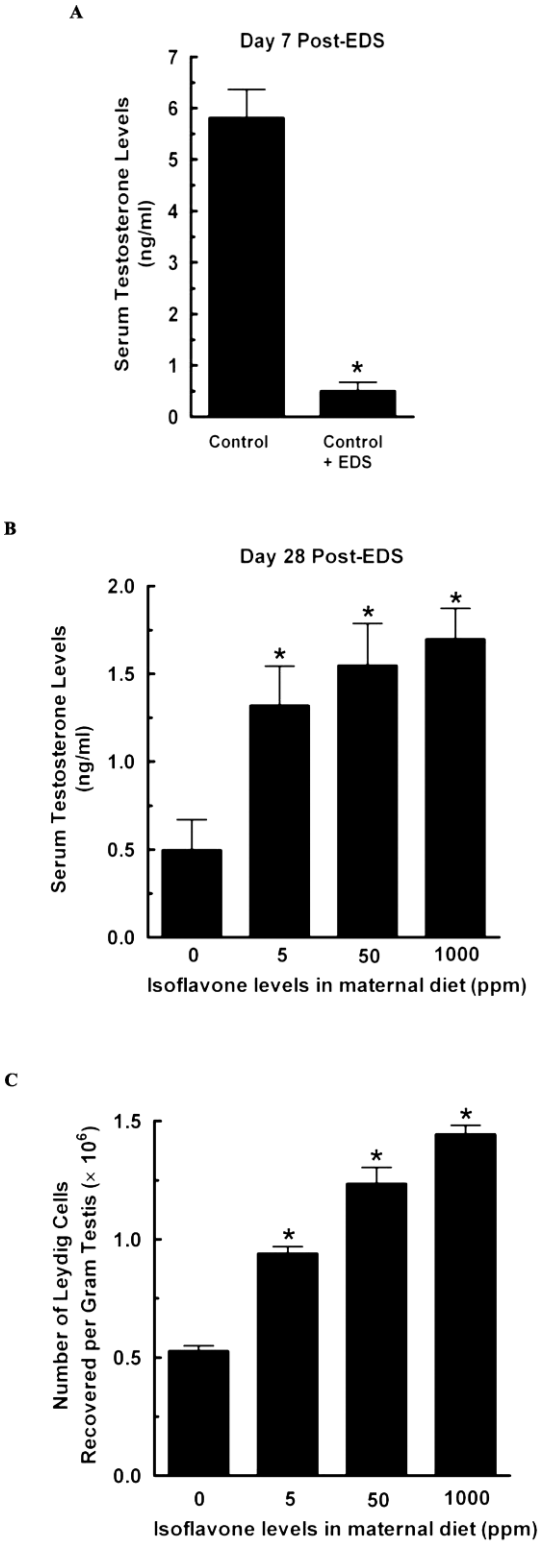

FIG. 6. Serum testosterone concentrations were measured as a marker for Leydig cell repopulation of testis following administration of the cytotoxin ethane dimethylsulfonate (EDS) to 60-day-old rats exposed to dietary isoflavones in the perinatal period. Serum testosterone levels were measured 7 days (**A**) and 28 days (**B**) after EDS administration. The numbers of Leydig cells recovered from testis of male rats exposed to dietary isoflavones compared to control animals was determined in sexually mature rats at 90 days (n = 9–10; **C**). *$P < 0.05$ versus control.

(Fig. 8D). Furthermore, exposure of male rats to isoflavones increased MAPK3/1 phosphorylation (p-MAPK3/1 or p-p42/44) in Leydig cells (Fig. 9A) ($P < 0.05$). A physiological role for MAPK3/1 in the regulation of Leydig cell division was suggested by measurement of greater p-MAPK3/1 levels in highly proliferative progenitor Leydig cells than in immature and adult Leydig cells (Fig. 9B) ($P < 0.05$). Involvement of MAPK in the mitogenic action of genistein was confirmed by results of inhibition studies whereby pretreatment of Leydig cells with the specific MAPK inhibitor PD 98095 blocked genistein induction of proliferative activity (Fig. 9C) ($P < 0.05$). Furthermore, pretreatment of Leydig cells with AKT kinase inhibitor or the antiestrogen ICI 182,780 abrogated genistein activation of MAPK3/1 (Fig. 9, D and E). Also, pretreatment with ICI 182,780 had the effect of abrogating genistein-induced AKT activation (Fig. 9F). Together, results of the inhibition studies demonstrated that the effect of genistein in Leydig cells was abrogated by AKT kinase inhibitor as well as by the antiestrogen ICI 182,780, suggesting genistein-induced interaction between AKT- and ESR-mediated pathways.

Perinatal Exposure to Soy Isoflavones Impacts Maturation of Steroidogenic Capacity in Leydig Cells

At 90 days of age, serum testosterone levels were unchanged in male rats exposed to the 5- and 50-ppm diets, but the levels were increased in the 1000-ppm diet group (Fig. 10A) ($P < 0.05$). Leydig cell testosterone production was unaffected in animals exposed to the 5- and 50-ppm diets but was decreased in the 1000-ppm diet group (Fig. 10B) ($P < 0.05$). Although not always reflected in measurements of testosterone secretion, exposure to isoflavones affected development of steroidogenic capacity in Leydig cells, because the levels of STAR (Fig. 10, C and D) and enzyme protein expression (i.e., CYP11A1 [Fig. 10, C and E], HSD3B [Fig. 10, C and F], and CYP17A1 [Fig. 10, C and G]) were increased, whereas levels of HSD17B3 were decreased (Fig. 10, C and H) ($P < 0.05$).

DISCUSSION

The present results demonstrate that soy isoflavones stimulate proliferative activity in developing Leydig cells at concentrations approximating those achieved after consumption of soy-based diets. This finding has implication for the use of soy-based diets by pregnant mothers and in infant formulas. For example, genistein and daidzein were detected, measuring in the nanomolar range, in amniotic fluid obtained from pregnant mothers [38, 39]. Also, approximately 750 000 infants fed soy-based cereals in the United States each year are exposed to genistein and daidzein, which attain blood concentrations in the range between 300 and 600 nM [40, 41]. These blood isoflavone levels can be achieved by consumption of diets containing isoflavones at concentrations between 30 and 1000 mg/kg diet [42]. Induction of proliferative activity was linked to increased expression of cell-cycle proteins, in agreement with reports indicating that mitotically active Leydig cells express high levels of proteins that support cell division [43, 44]. For example, PCNA is known to function as a cofactor of DNA polymerase during DNA synthesis in the nucleus [45], and phosphorylation of RB on serine residue 780 activates genes required for cell-cycle progression [46]. Our findings agree with suggestions that low concentrations of genistein ($\leq$10 μM) tend to induce cell

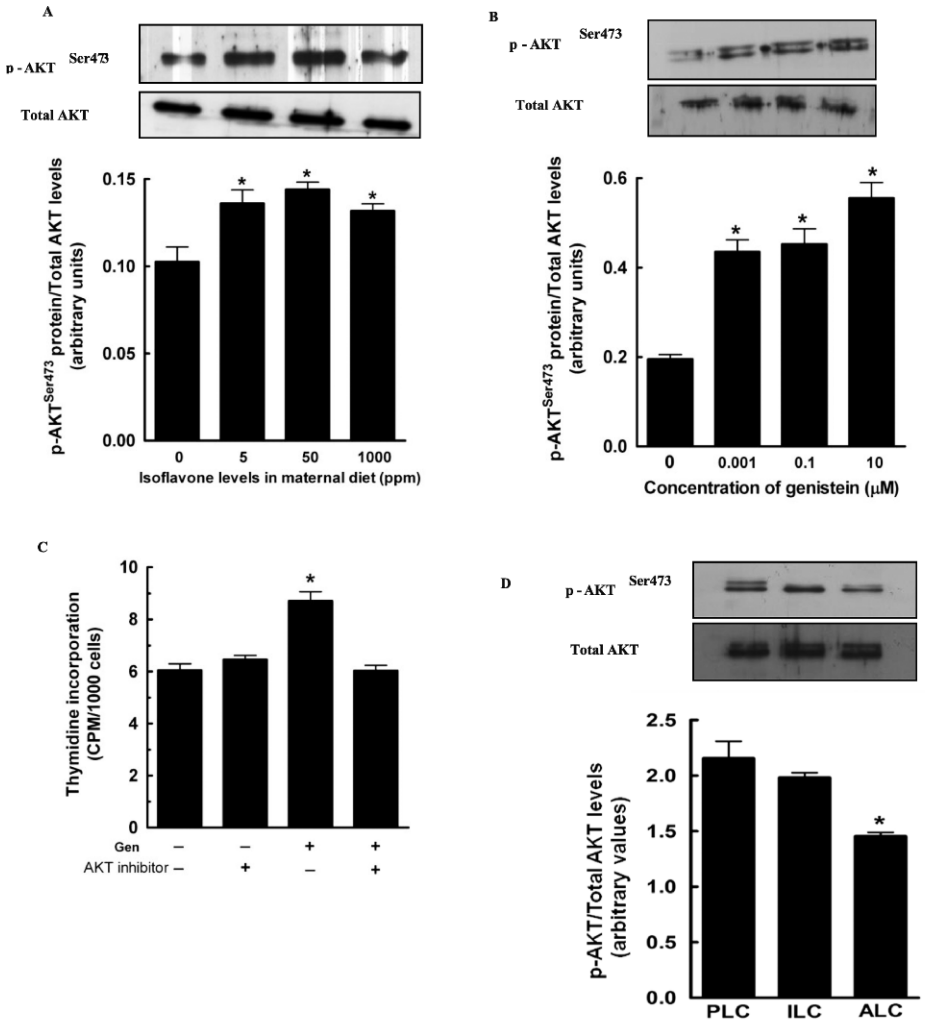

FIG. 7. Phosphorylation of protein kinase B or AKT on serine residue 473 (pAKT^{Ser473}) in Leydig cells was assessed by immunoblot analysis in Leydig cells after exposure to dietary isoflavones in vivo (**A**) or genistein treatment in vitro (**B**). Cellular [^{3}H] thymidine uptake was analyzed in genistein (Gen)-treated Leydig cells (3 h) after pretreatment with AKT kinase inhibitor for 1 h (**C**). Developmental changes in AKT phosphorylation (pAKT^{Ser473}) were analyzed in freshly isolated progenitor (21 days; PLC), immature (35 days; ILC) and adult Leydig cells (90 days; ALC) (**D**). Immunoblots are representative of at least four separate Western blot procedures for animal studies (**A** and **D**). Experiments using isolated Leydig cells were performed at least three times using triplicate cultures of Leydig cells per group per experiment (**B** and **C**). The pAKT levels were normalized to total (inactive) AKT levels. AKT = 56 kDa, pAKT = 60 kDa. *$P < 0.05$ versus control or PLC.

proliferation in estrogen-sensitive tissues [47–51]. As in the present study, increases in serum testosterone levels were previously associated with decreased steroidogenic enzyme activity, indicating the possibility of greater Leydig cell numbers, especially as animals were fed soy diets beginning in the prepubertal and pubertal periods [42, 52] of intense mitotic activity in Leydig cells [19]. Our observations are also in agreement with reports of increased Leydig cell numbers in marmoset monkeys raised on soy-based formula [53].

Perinatal exposures to isoflavones affected differentiation of Leydig cell steroidogenic capacity, as evidenced by altered expression of proteins involved in androgen biosynthesis (i.e., STAR and steroidogenic enzymes). Although STAR protein levels were increased in the present study, we observed previously that such increases may, in fact, be caused by decreased STAR phosphorylation, which is required for androgen biosynthesis [14, 54]. Protein expression was increased for several steroidogenic enzymes, but expression of HSD17B3 was markedly decreased. The HSD17B3 enzyme catalyzes the final enzymatic step in androgen biosynthesis, which converts androstenedione to testosterone. Similar reports have appeared linking ESR1-mediated activity to inhibition of *Cyp17a1* and *Hsd17b3* gene expression in the mouse ovary [55, 56]. Therefore, decreased androgen biosynthesis likely resulted from disruption of STAR function and HSD17B3 activity. Nevertheless, this interpretation does not preclude the possibility that other sites in the androgen biosynthetic pathway are subject to interference by isoflavones. In this regard, we recently observed that genistein caused direct inhibition of HSD3B activity in the human and rat testis [57]. Thus, perinatal exposures to soy isoflavones possibly exert differential effects on steroidogenic enzyme capacity in Leydig cells. Moreover, genistein is known to exert other biological effects, including tyrosine kinase inhibition and antioxidant activity [58, 59]. Additional studies are required to determine how these factors influence genistein regulation of Leydig cells.

Interestingly, previously observed differences in body and testis weights between isoflavone-exposed and control animals [14] were not seen in the present study. This disparity prompted us to perform experiments investigating the potential for soy-based diets to alter metabolism and affect body weight. Available results showed that serum adiponectin levels were greater in isoflavone-exposed rats than in control animals (our unpublished data). Among other effects, adiponectin is known to possess insulin-sensitizing properties [60]. Thus, the exposure paradigms used in our studies probably do not provoke profound and sustainable changes in body weight. Also, we failed to observe any enlargement of accessory sex glands in animals exhibiting increased serum testosterone levels. However, the lack of sex organ enlargement may be caused by a number of factors: 1) Exposure to increased serum testosterone concentrations probably required more time for sex gland enlargement to become apparent, 2) isoflavone-induced decreases in ESR1 [14] and AR [61] protein may

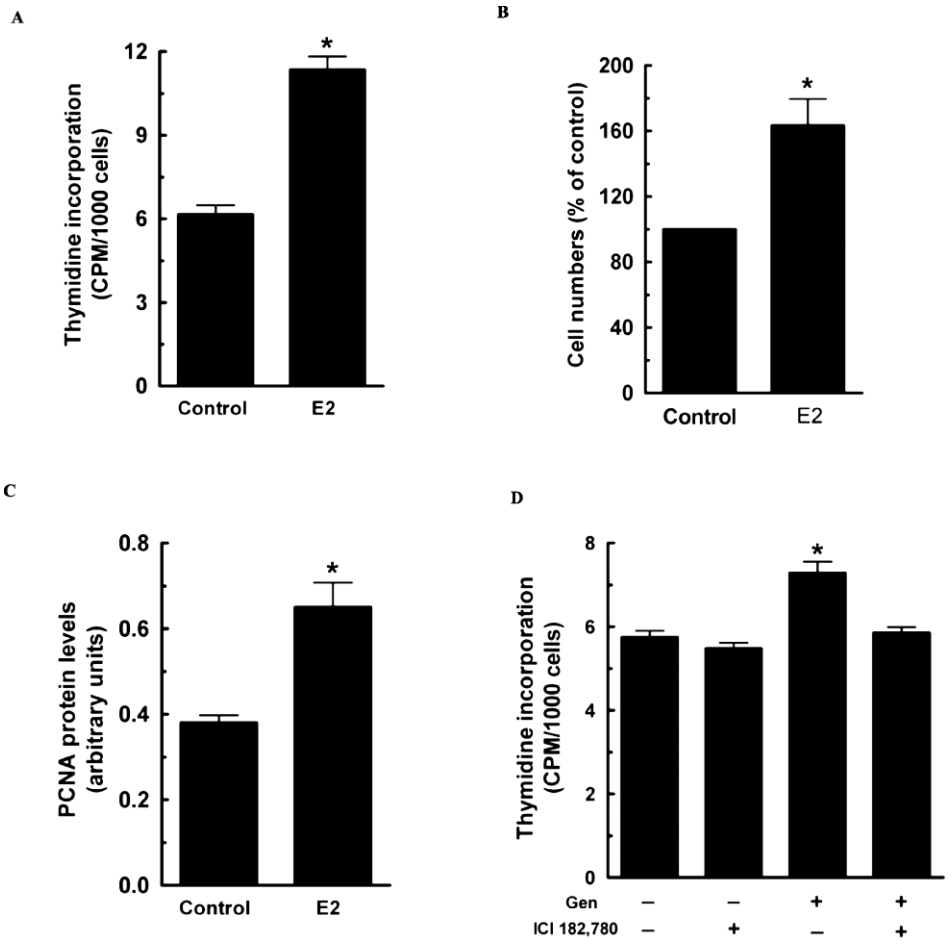

FIG. 8. Proliferative active activity was analyzed in progenitor Leydig cells incubated with E_2 (10 nM) by cellular [^{3}H]thymidine uptake (**A**), cell numbers (**B**), and PCNA expression (**C**). The levels of PCNA (36 kDa) were normalized to β-actin (ACTB; 42 kDa). Leydig cells were pretreated with the pure antiestrogen ICI 182,780 and subsequently were incubated in culture medium containing genistein (Gen) followed by analysis of cellular [^{3}H]thymidine uptake (**D**). *$P < 0.05$ versus control.

mitigate androgen stimulation, and 3) although 5α-reductase enzyme activity was not investigated, conversion of testosterone into the more potent dihydrotestosterone may be required to achieve tissue hypertrophy [62].

The present observations indicating that exposures to isoflavones affect Leydig cell division and steroidogenesis are, perhaps, not entirely surprising. Leydig cells express ESRs, and soy isoflavones can activate ESRs [24, 25]. However, it is intriguing to note that exposures to the 5- and 50-ppm diets induced proliferative activity in Leydig cells to approximately the same degree as exposure to the 1000-ppm diet. The reasons for this comparable effect over a wide dose range are not clear. It is possible that enhanced Leydig cell proliferation caused by the 5- and 50-ppm diets is associated with upregulation of LHR, AR, and ESR1 protein. Changes in gene expression levels for transcription factors are thought to amplify or diminish hormone-stimulated signaling [26, 27]. Also, it is interesting to note that changes in hormone receptor protein expression (LHR, AR, and ESR1), as with anogenital distance measurements, were for the most part larger in the 5-ppm and/or 50-ppm diet groups compared to the control and 1000-ppm groups. This pattern of effects caused by exposure to a wide range of doses of agents with estrogenic activity is classified as a ''nonmonotonic dose response,'' and it is characterized by biological responses that decrease at doses above those that initially reach a level of saturation [63].

Serum E_2 levels were increased in male rats exposed to isoflavones, as has been reported in mice [64] and human subjects maintained on soy diets [61]. Aromatase is the enzyme that catalyzes E_2 biosynthesis using testosterone as substrate. Therefore, increased serum E_2 levels are possibly the result of greater substrate availability. Although not measured in the present study, increased serum E_2 may also be related, at least in part, to enhanced aromatase activity. In this regard, adipose tissue, which is the predominant source of serum E_2 in both males and females, expresses aromatase and ESRs and is a direct target for stimulation by ESR agonists. Thus, soy isoflavones may bind ESRs in adipose tissue to regulate E_2 biosynthesis [65]. Given that E_2 is a potent mitogen, increased serum E_2 levels probably were a factor in isoflavone-induced stimulation of Leydig cell proliferation. This line of thinking is supported by reports showing that genistein caused mammary gland hyperplasia in the male rat [66] and that elevated serum E_2 levels were associated with gynecomastia in an adult male subject who had consumed unusually large amounts of soy milk [67].

The present results show that the mitogenic action of genistein is mediated by ESRs, acting in concert with AKT and MAPK. Although rat Leydig cells express only ESR1, genistein is known to bind both ESR1 and ESR2 [31, 32], which have been localized to various cell types in the rat, mouse, and human testis [68–72]. However, in contrast to the widely known action of estrogen in Leydig cells, little information is available regarding AKT, which shares significant homology with protein kinases A and C [73]. Nevertheless, profound decreases in AKT1 and AKT2 mRNA levels were associated with the transition from progenitor to adult Leydig cells in the rat [74]. Also, AKT is thought to be an antiapoptotic factor, because wortmannin, an inhibitor of phosphatidylinositol 3-kinase, which acts upstream of AKT,

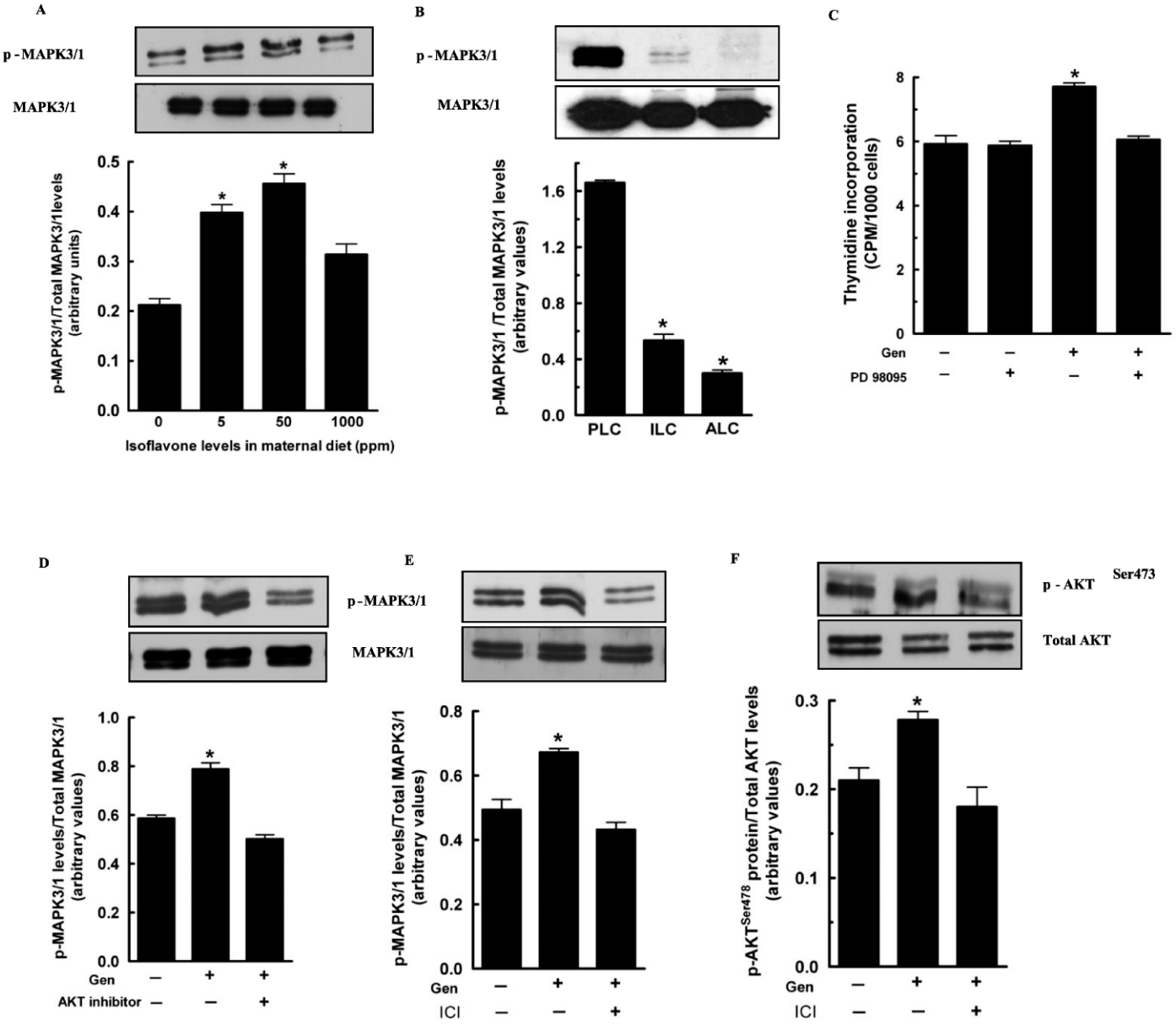

FIG. 9. Activation of extracellular regulated kinase (MAPK3/1; i.e., p-MAPK3/1 or p-p42/44) was analyzed in Leydig cells isolated from male rats exposed to dietary isoflavones (**A**). Developmental changes in p-MAPK3/1 activation were analyzed in progenitor Leydig cells (21 days; PLC), immature Leydig cells (35 days; ILC), and adult Leydig cells (90 days; ALC) (**B**). Leydig cells were assessed for [^{3}H]thymidine uptake after pretreatment with the specific MAPK inhibitor (PD 98095) followed by incubation in culture medium containing genistein (Gen; **C**). Activation of MAPK3/1 was analyzed in genistein-treated Leydig cells (3 h) after pretreatment with AKT inhibitor (**D**) and the antiestrogen ICI 182,780 (ICI; **E**) for 1 h. Phosphorylation of AKT on serine residue 473 (pAKT^{Ser473}) was analyzed in genistein-treated Leydig cells after pretreatment with ICI 182,780 (**F**). Data from in vitro experiments represent results from two or three separate and independent experiments. Immunoblots are representative of at least four separate Western blot procedures from each experiment. The levels of p-MAPK3/1 (p-p42/44) were normalized to total MAPK3/1 (p-42/44) levels. Genistein, AKT inhibitor, and ICI 182,780 were used at 0.1 μM. *$P < 0.05$ versus control.

abrogated AKT phosphorylation and the antiapoptotic effect of insulin-like growth factor 1 in immature Leydig cells [75]. In the present study, we obtained two pieces of evidence implying a role for AKT in the regulation of Leydig cell division. First, the levels of pAKT^{Ser473} were greater in proliferating Leydig cells after in vivo and in vitro exposure to isoflavones. Second, age-dependent decreases occurred in pAKT^{Ser473} levels during the transition from progenitor to adult Leydig cells. These findings tend to support the general view that the signaling pathway mediated by phosphatidylinositol 3-kinase and AKT regulates survival signals and enhances cellular resistance to programmed cell death [30, 76–78]. Therefore, AKT is likely a target for mitogenic factors regulating Leydig cells during the peripubertal period of reproductive tract development.

The finding that the action of genistein in Leydig cells was abrogated equally by the AKT kinase inhibitor and the antiestrogen ICI 182,780 seems to imply that molecules in the signaling pathways mediated by the ESR and AKT are involved in genistein regulation of Leydig cell division, and it suggests that MAPK possibly facilitates cross-talk between ESR- and AKT-mediated pathways. Cross-talk between signaling pathways in estrogen-sensitive tissues is thought to synergize and enhance plasticity of estrogen action [79]. In this regard, we obtained data indicating that MAPK3/1 are involved

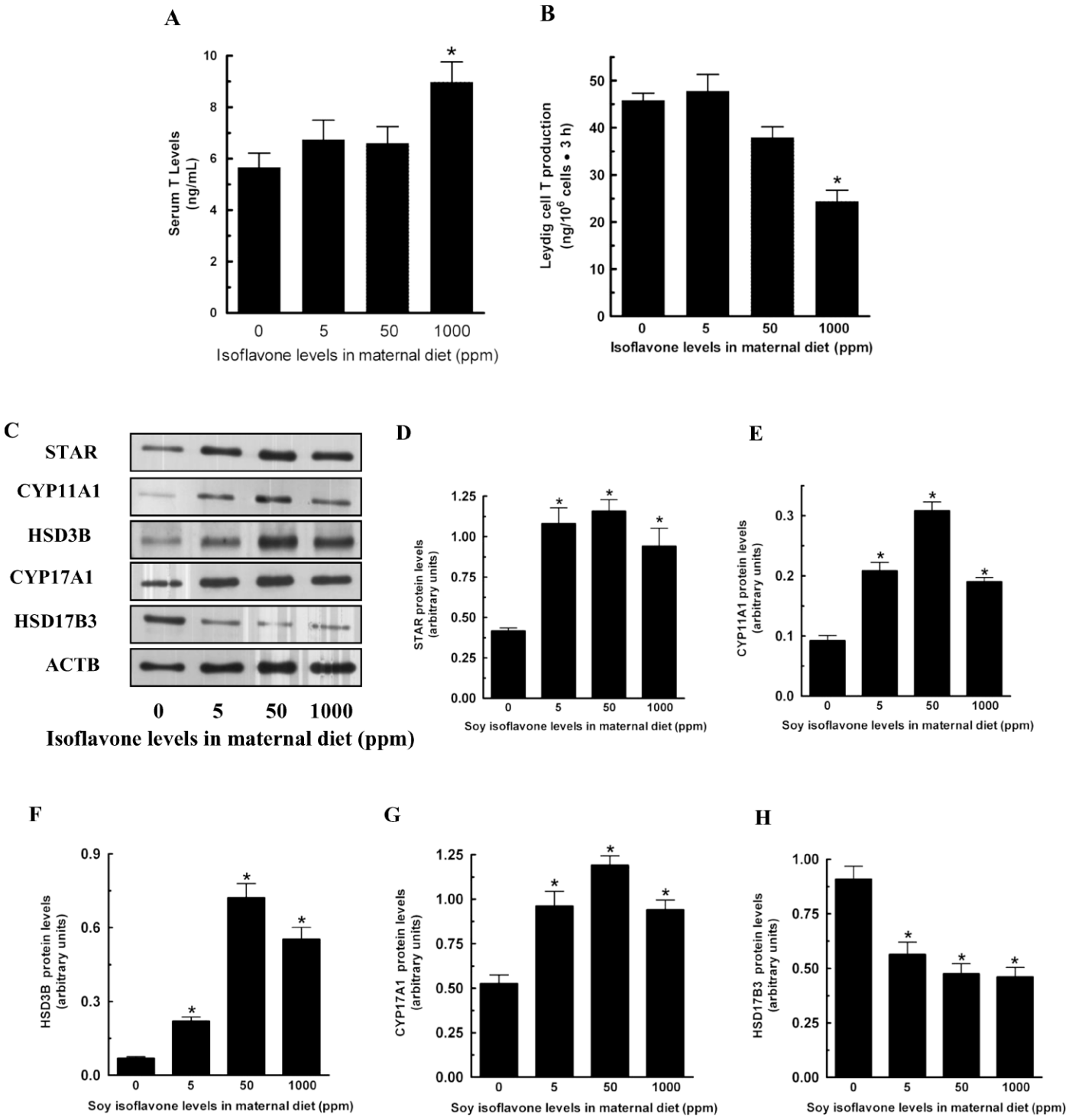

FIG. 10. Serum and Leydig cells were collected from adult male rats exposed to dietary isoflavones in the perinatal period. Serum was collected from a total of 32 to 35 animals per group, whereas Leydig cells were isolated from 9 to 10 animals per group and incubated in triplicate in culture medium for 3 h. The concentrations of testosterone (T) in serum (**A**) and aliquots of spent media (**B**) were assayed in duplicate by radioimmunoassay. Protein expression levels for STAR and enzymes involved in androgen biosynthesis were assessed in whole-cell lysates by Western blot analysis using anti-STAR (**C** and **D**), anti-CYP11A1 (**C** and **E**), anti-HSD3B (**C** and **F**), anti-CYP17A1 (**C** and **G**), anti-HSD17B3 (**C** and **H**) antibodies, and the appropriate secondary antibodies. Data represent results from at least four Western blot procedures per experiment, and enzyme protein levels were normalized to β-actin (ACTB). STAR = 30 kDa, CYP11A1 = 60 kDa, HSD3B = 42 kDa, CYP17A1 = 55 kDa, HSD17B3 = 35 kDa, ACTB = 42 kDa. *$P < 0.05$ versus control.

in the mitogenic action of genistein, thereby providing a putative mechanism for cross-talk between AKT- and ESR-mediated signaling. This interpretation is in line with suggestions that genistein binds to ESRs and causes downstream activation of AKT, which in turn activates MAPK3/1. Alternatively, genistein may act independently, albeit simultaneously, through ESR- and AKT-mediated pathways, which then converge on MAPK3/1 as downstream effector molecules [33, 80]. Indeed, it has been proposed that the biphasic effect of genistein on cell division results from differential MAPK

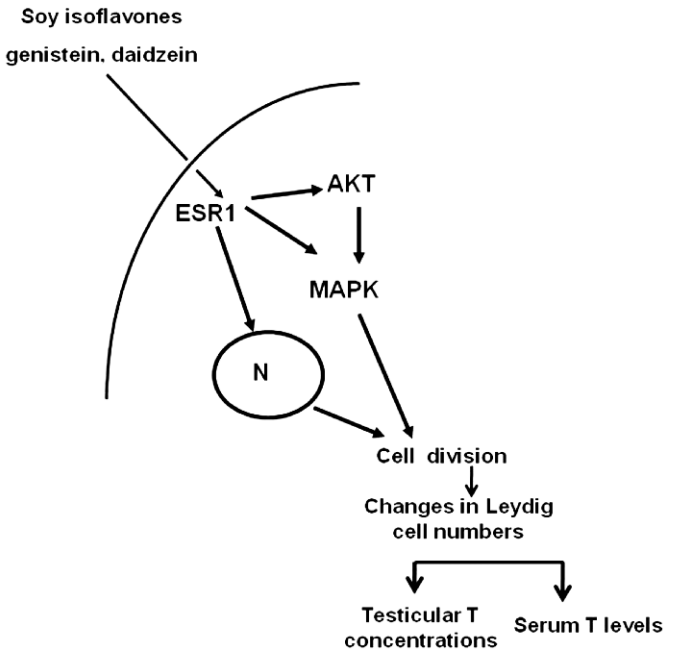

FIG. 11. Pathways regulating Leydig cell division activated by soy isoflavones. Present results indicate that the mitogenic action of genistein is mediated by estrogen receptors acting in concert with the phosphatidylinositol 3-kinase/AKT (PIK3/AKT) signal transduction pathway and the pathway mediated by MAPK. ESR-genistein complexes may act through estrogen-response elements in the nucleus to regulate gene expression and/or induce protein-protein interactions in the PIK3/AKT- and MAPK-mediated signaling pathways to influence kinase activation affecting cell-cycle progression. Modulation of proliferative activity in developing Leydig cells potentially alters the number of Leydig cells in the adult testis and affects intratesticular and serum testosterone (T) levels. N, nucleus.

activity, with low concentrations stimulating ESR-mediated cell growth and proliferation, which involves MAPK activation, and higher concentrations (>10 μM) inducing non-ESR-mediated pathways and causing downstream inactivation of MAPK to decrease the rate of mitosis [81–83]. Overall, the present data indicated that ESR1 caused direct activation of MAPK3/1 or activated AKT, which acts upstream of MAPK3/1, to accelerate cell division and, thereby, alter Leydig cell numbers (Fig. 11).

Perhaps the most significant finding in the present study is the observation that isoflavones increased Leydig cell numbers, which enhanced serum and testicular testosterone concentrations even as androgen biosynthesis was declining. Although genistein concentrations of 10 μM or greater are thought to decrease cell proliferation [47–51], regular feeding with soy infant formulas will not likely produce tissue concentrations in excess of 10 μM. Thus, exposures to isoflavones occurring in the fetal and neonatal periods have the potential to alleviate affects associated with deficits in androgen biosynthesis and/or augment testicular and serum testosterone levels. This effect has implications for reproductive health, because high intratesticular testosterone concentrations may adversely affect the process of spermatogenesis and sperm production. For example, suppression of spermatogenesis by exogenous administration of testosterone, which increases intratesticular levels, has been explored for development of a male contraceptive [84]. Also, high intratesticular testosterone was found to suppress spermatogonial proliferation in the radiation-damaged rat testis [85]. Other reports have established that elevated serum testosterone levels increased the risk for testicular germ cell tumors in human subjects [86, 87]. On the other hand, a number of studies have shown that isoflavones may act within the seminiferous epithelium to impact spermatogenesis without concomitant endocrine changes [88, 89]. Thus, multiple cell types in the testis are likely targets for the action of isoflavones. Also, the testicular actions of daidzein and equol, which are present in body tissues (along with genistein) after consumption of soy-based meals, have received little or no attention. Additional studies are warranted to examine the outcome of combined action by isoflavones in Leydig cells and germ cells. These studies will provide information relevant to risk assessment of the population regarding the potential for soy-based food products to cause testicular toxicity, especially in neonates.

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