Estradiol Induction of Spermatogenesis Is Mediated via an Estrogen Receptor-α Mechanism Involving Neuroendocrine Activation of Follicle-Stimulating Hormone Secretion

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Both testosterone and its nonaromatizable metabolite dihydrotestosterone (DHT) induce spermatogenesis in gonadotropin-deficient *hpg* mice. Surprisingly, because aromatization is not required, estradiol (E2) also induces spermatogenesis and increases circulating FSH in *hpg* mice, but the mechanism remains unclear. We studied E2-induced spermatogenesis in *hpg* mice on an estrogen receptor (ER)-α (*hpg*/αERKO) or ERβ (*hpg*/βERKO) knockout or wild-type ER (*hpg*/WT) background treated with subdermal E2 or DHT implants for 6 wk. In *hpg*/WT and *hpg*/βERKO, but not *hpg*/αERKO mice, E2 increased testis and epididymal weight, whereas DHT-induced increases were unaffected by ERα or ERβ inactivation. E2 but not DHT treatment increased serum FSH (but not LH) in *hpg*/WT and *hpg*/βERKO but not *hpg*/αERKO *hpg* mice. DHT or E2 alone increased (premeiotic) spermatogonia and (meiotic) spermatocytes without significant change in Sertoli cell numbers. DHT alone increased postmeiotic spermatids, regardless of ER presence, compared with variable ERα-dependent E2 postmeiotic responses. An ERα-mediated effect was confirmed by treating *hpg* mice for 6 wk by subdermal selective ER-α (16α-LE$_2$) or ERβ (8β-VE$_2$) agonist implants. ERα (but not ERβ) agonist increased testis and epididymal weight, Sertoli cell, spermatogonia, meiotic, and postmeiotic germ cell numbers. Only ERα agonist markedly increased serum FSH, whereas either agonist induced small rises in serum LH. Administration of ERα agonist or E2 in the presence of functional ERα induced prominent gene expression of specific Sertoli (*Eppin*, *Rhox5*) and Leydig cell (*Cyp11a1*, *Hsd3b1*) markers. We conclude that E2-induced spermatogenesis in *hpg* mice involves an ERα-dependent neuroendocrine mechanism increasing blood FSH and Sertoli cell function. **(*Endocrinology* 151: 2800–2810, 2010)**

Both individual and synergistic actions of testosterone (T) and FSH are required for optimal regulation of spermatogenesis (1–3). The concerted roles of FSH and T are mediated via Sertoli cells, which express the FSH and androgen receptor (AR), both of which are absent in developing male germinal cells. The hormonal actions of each have been illustrated clearly by phenotypes exhibited in mouse models null for the respective hormone pathways. For example, hypogonadal (*hpg*) mice are functionally deficient in gonadotrophins and sex steroids due to a naturally occurring deletion in the *Gnrh1* gene (4, 5) and consequently exhibit immature testis with spermatogenic arrest at the pachytene stage of meiosis (6). Disruption of FSH action alone, by either inactivation of the FSHβ subunit (7) or receptor (8, 9) genes, leads to reduced sperm production and mature testis size due primarily to an in-

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Abbreviations: AR, Androgen receptor; DHT, dihydrotestosterone; E2, estradiol; ER, estrogen receptor; ERKO, knockout lacking ER; *hpg*, hypogonadal; qPCR, quantitative PCR; T, testosterone; WT, wild type.

sufficient Sertoli cell population (4, 5). Whereas FSH actions alone can maintain Sertoli cell number (10), androgen actions are indispensable for the completion of postmeiotic germ cell development (6). This critical role for androgen signaling is consistent across mouse models with defective androgen signaling globally (11) or in Sertoli cells only (12–15), with spermatogenesis blocked at the tetraploid pachytene spermatocytes stage. Hence, there is abundant evidence that induction of spermatogenesis depends on the complementary actions of FSH and androgens, the former to establish a sufficient Sertoli cell population and the latter for functional completion of meiosis and postmeiotic sperm differentiation/maturation.

Although an absolute requirement for androgen action in spermatogenesis is clear, the precise molecular mechanisms involved in steroid-induced testicular development are not. Qualitatively complete spermatogenesis can be restored in *hpg* mice by administration of either T or its metabolite, the potent nonaromatizable androgen dihydrotestosterone (DHT), in the absence of FSH stimulation (6). These data strongly indicate that the effects are exerted directly on the testis, did not require aromatization, and were mediated via the AR (6). However, later studies demonstrated that comparable spermatogenesis in *hpg* mice is induced after similar treatment with estradiol (E2) (16). Interestingly, this E_2 effect is dependent on a functional AR (17, 18) and coincides with increased circulating FSH levels. Prompted by the disparities in the available evidence on the mechanism and site of action by which E2 induces spermatogenesis in *hpg* mice, the present study set out to segregate the spermatogenic actions of E2 by two complementary pharmacogenetic experiments using: 1) selective estrogen receptor (ER)α or ERβ agonists in gonadotropin-deficient *hpg* mice, and 2) pure androgen (DHT) and estrogen (E2) treatments in novel compound *hpg*/ER knockout lines selectively lacking ERα or ERβ (*hpg*/αERKO or *hpg*/βERKO mice) on the *hpg* genetic background. Noting that spermatogenesis occurs normally in mice rendered estrogen deficient due to genetic disruption of estradiol synthesis (19) or either of its ERs (20–23), our results from this *in vivo* approach reveal that E2-induced spermatogenesis in hypogonadotrophic mice is dependent on functional ERα but not ERβ.

Materials and Methods

Mice

For study 1, *hpg* mice (*Gnrh1*$^{-/-}$ males, C3H/HeH × 101/H) were maintained under standard housing conditions (*ad libitum* access to food and water in a temperature and humidity controlled, 12-h light cycle environment) at the ANZAC Research Institute. All experiments were approved by the Sydney South West Area Health Service's Animal Welfare Committee within National Health and Medical Research Council ethical guidelines for animal experimentation. Genotype was confirmed by PCR (24). All procedures were performed under ketamine/xylazine anesthesia using additional analgesics if required for nonterminal procedures.

For study 2, *hpg* mice were crossed onto the ERα knockout (αERKO) and ERβ knockout (βERKO) lines to generate hypogonadal forms of each respective ER-null line. Compound *hpg*/αERKO and *hpg*/βERKO mice were obtained from individual colonies that were first established by crossing heterozygous *Gnrh1*$^{+/-}$ males (C3H/HeH × 101/H; Jackson Laboratory, Bar Harbor, ME) with heterozygous *Esr1*$^{+/-}$ (αERKO) or *Esr2*$^{+/-}$ (βERKO) females (C57BL/6) to generate compound heterozygous animals for general breeding. The *hpg* (*Gnrh1*$^{-/-}$) littermate males expressing native ERα and ERβ (termed *hpg*/WT) derived from these heterozygous breeding pairs were considered comparable and used as controls. All animals were genotyped for targeted *Esr1* or *Esr2*, and mutant *Gnrh1* alleles as described previously (25) by PCR on DNA extracted from tail biopsies using the Wizard SV 96 Genomic DNA extraction kit (Promega, Madison, WI). Mice were individually identified by an implanted microchip transponder (BioMedic Data Systems, Seaford, DE) and maintained in plastic cages in a temperature-controlled room (21–22 C) under a 12-h light, 12-h dark schedule with access to NIH 31 mouse chow and fresh water *ad libitum*. Mice were killed by exsanguination under anesthesia with a sublethal dose of sodium pentobarbital. The Animal Care and Use Committee of the National Institute of Environmental Health Sciences preapproved all procedures involving animals.

Drugs

ERα (16α-LE_2) or ERβ (8β-VE_2) agonists were provided by Bayer Schering Pharma (previously Schering, Berlin, Germany) (26, 27). Drug treatments were administered by subdermal implantation of 60-d release pellets (Innovative Research Associated, Sarasota, FL) containing the ERα agonist (16α-LE_2, 15 and 45 μg/kg · d, nominally 0.375 and 1.125 μg per 25 g mouse per day) or ERβ agonist (8β-VE_2, 150 and 450 μg/kg · d, nominally 3.75 and 11.25 μg per 25 g/mouse · d). Doses used were previously shown to induce maximal ER-specific effects of each agonist (28).

E2 and DHT were administered via subdermal implantation of single SILASTIC brand tubes (inner diameter 1.47 mm, outer diameter 1.95 mm, end sealed with SILASTIC brand adhesive; Dow Corning Corp., Midland, MI) filled to 1 cm with crystalline DHT (6) or E2 recrystallized into a homogenous powder after dilution (1:1000) with cholesterol (18). Control animals received no implant.

Study design

In study 1, weanling (3 wk old) *hpg* males (n = 11–23/group) were untreated or treated for 6 wk with selective ERα (16α-LE_2) or ERβ (8β-VE_2) agonists. Untreated age-matched wild-type littermate males were used as controls. After 6 wk of treatment, mice were weighed and blood collected (processed serum stored at −20 C) under anesthesia by cardiac exsanguination; then one testis was immediately excised, weighed, and snap frozen in liquid nitrogen. Animals were then perfused with heparinized 0.9% saline followed by Bouin's fixative via the left ventricle and the fixed contralateral testis collected for sectioning and stereological evaluation; prostate lobes (anterior, ventral, dorsolateral), epididymis, seminal vesicles, spleen, and pituitary were dissected and weighed.

In study 2, E2 or DHT induction of spermatogenesis was examined in *hpg*/WT, *hpg*/αERKO, and *hpg*/βERKO mice (n = 7–25/group) of 6–12 wk of age administered single subdermal SILASTIC brand implants (1 cm; Dow Corning) containing either crystalline E2 or DHT. Control animals received no implant. Due to limited animal availability, mice were treated in small cohorts, each cohort consisting of both control and treated littermate mice, and data then combined for analysis. Six weeks after implantation, mice were anesthetized and weighed, serum collected from retroorbital bleed and then perfused, and tissues collected as described in study 1. Weights of testes, epididymis, seminal vesicles, liver, spleen, heart, and kidneys were recorded and serum stored at −20 C.

Histology and stereology

After whole-body perfusion with modified Bouin's fixative, testes were dissected and placed in fresh Bouin's solution for an additional 24 h and then transferred to 70% ethanol and processed for histological examination. Sertoli and germ cell populations were quantified stereologically by the optical dissector technique as described previously (3, 29) using CASTGRID (Olympus, Aarhus, Denmark) software.

Assays

Mouse serum FSH (30) and LH (31) levels were measured by immunofluorometric assays as described previously. Serum testosterone, DHT, and E2 were measured in a single assay batch using a novel liquid chromatography, tandem mass spectrometry method as described (32) as adapted for mouse samples (33). Briefly, 0.1 ml serum was extracted with hexane-ethyl acetate fortified with deuterated internal standards and run without derivatization. The limits of quantification were 0.1 ng/ml, 0.2 ng/ml, and 5 pg/ml, respectively.

Gene expression

Total RNA from flash frozen testes was obtained after homogenization in TRI reagent (Sigma, St. Louis, MO) or TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Testis mRNA expression levels of Sertoli cell-specific *Eppin* (also known as *Spinlw1*) and *Rhox5*, and Leydig cell *Cyp11a1* and *Hsd3b1* were quantified by real-time quantitative PCR (qPCR) analysis of cDNAs using a Corbett RotorGene 6000 (Corbett Research, Sydney, Australia) and validated primers [*Eppin* and *Rhox5* (15); *Cyp11a1* (34); murine *Hsd3b1* (SA Biosciences, Frederick, MD)] with RT2 SYBR Green qPCR master mix (SA Biosciences). Mouse *Wbscr1* primers were used as a housekeeping gene and loading control to standardize relative transcript expression levels in different samples (n = 4–10/group) as described (15). Previous work showed that expression level of *Wbscr1* is relatively high and consistent during murine testicular development, indicating its suitability as a housekeeping gene expressed at similar levels in somatic and germ cell populations (35).

To estimate total Sertoli or Leydig cell transcript expression levels in whole-testis RNA in which testis size differed according to treatments in each study, a two-step adjustment procedure was used. First, an adjustment for the housekeeping gene is required to correct for differences in loading of the cDNA on a per-cell basis in the qRT-PCR. By definition, this housekeeping gene is expressed to a similar degree in all cells, including germinal and nongerminal cells. This first adjustment provides a valid estimate of gene expression level in whole-testis RNA.

Because induction of spermatogenesis causes an intense proliferation of germ cells, this greatly dilutes the measured gene expression levels of the nonproliferating, specialist somatic cells when estimated from whole-testis RNA. To estimate the total RNA level in Sertoli (or Leydig) cells therefore requires a second adjustment to extrapolate the per-cell estimate from whole-testis RNA to Sertoli (or Leydig) cell RNA. This adjustment multiplies the whole-cell RNA by the tissue/cell proportion represented by the Sertoli (or Leydig) cells to obtain the total RNA in each of these target cells. Consequently, a correction factor to adjust for the dilution of nonproliferating somatic cell RNA by the proliferation of germinal cells is best approximated by the ratio of testis weights because germinal epithelium comprises the bulk (85%) of testis volume (5). This procedure is valid for expression of Sertoli or Leydig cell-specific genes because neither Sertoli (this study) nor Leydig cell (36) numbers in the testis are changed by exogenous sex steroid treatments. Therefore, this second adjustment allows estimation of RNA levels for specific genes in the relatively constant somatic (Sertoli, Leydig) cell populations after correcting for the dilutional effect of the intense proliferation of germ cells as also reported previously (35). All samples were tested in duplicate.

Data analysis

Data were analyzed by one- and two-way ANOVA as appropriate to the study design with Tukey-Kramer and linear contrasts as *post hoc* tests as required using NCSS software (NCSS, Kaysville, UT). The magnitude of the DHT effect was compared between genotypes by using the pooled SE from suitable linear contrasts for DHT *vs.* no treatment. All data are expressed as mean ± SEM.

Results

Study 1

Male *hpg* mice were treated for 6 wk with selective ERα or ERβ agonists at low and high doses and then compared with untreated *hpg* mice. As shown in Fig. 1, the low (15 μg/kg) and high (45 μg/kg) doses of ERα agonist caused comparable increases in the weight of testis, seminal vesicle, anterior prostate lobe, and pituitary. Also increased in weight were the epididymis and ventral and dorsolateral prostate lobes, whereas spleen weight was unaffected and body weight decreased compared with values from untreated *hpg* animals (data not shown). In contrast, neither the low (150 μg/kg) nor high (450 μg/kg) doses of ERβ agonist had any effect on body or any organ weight examined (Fig. 1).

Both doses of ERα agonist equally and markedly induced serum FSH levels in *hpg* males (Fig. 1). The ERβ agonist also produced a significant rise in FSH ($P = 0.019$ *vs.* low dose, Fig. 1); however, this was observed in mice treated with the high dose only and was minimal com-

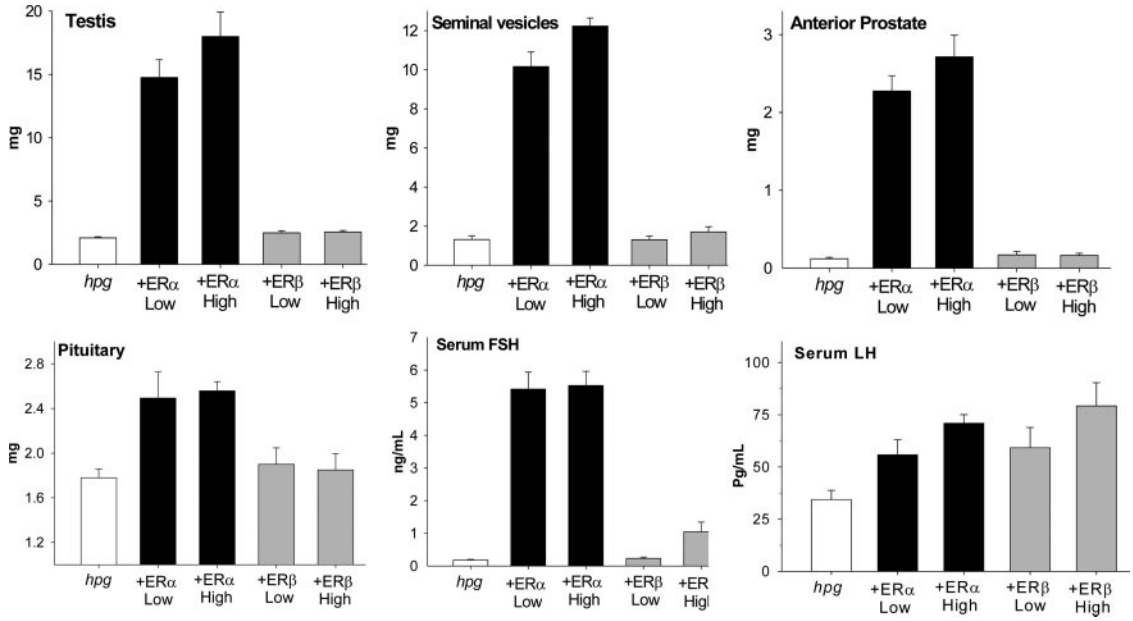

FIG. 1. Testis (*upper left*), seminal vesicle (*upper middle*), and anterior prostate lobe (*upper right*) weights together with pituitary (*lower left*), serum FSH (*lower middle*), and LH (*lower right*) panels in *hpg* mice treated with low or high doses of a selective ERα (*black bars*) or selective ERβ (*gray bars*) agonist or untreated (*white bars*). Data expressed as mean and SEM. For further details, see text.

pared with the effect of the ERα agonist. In contrast, small but significant increases in serum LH in *hpg* males were induced by both ERα or ERβ agonists, compared with untreated controls (Fig. 1).

As expected, testes of untreated *hpg* mice displayed the immature germinal epithelium characteristic of stalled postnatal meiotic development, including random distribution of immature Sertoli cells (Fig. 2). The ERα agonist (45 μg/kg) stimulated the formation of haploid spermatids with elongating nuclei in *hpg* testis (Fig. 2). The testes of ERα agonist-treated mice contained haploid spermatids with elongating nuclei and mature Sertoli cells properly organized within the germinal epithelium, typically located adjacent to the basement membrane and featuring tripartite nucleoli (Fig. 2). The ERα agonist treatment increased tubular diameter, although tubular lumina were absent or poorly developed, and hypertrophy and increased vesiculation of the Leydig cells compared with testes of untreated *hpg* mice. In contrast, neither dose of the ERβ agonist had an effect on testis morphology in *hpg* mice (Fig. 2C).

Stereological evaluation of the testes from the different treatment groups showed that the administration of ERα agonist (45 μg/kg) produced an increase in Sertoli cell number and significant increases in numbers of spermatogonia, spermatocytes (early and pachytene), and spermatids (round and elongated) (Fig. 2). The results were the same when expressed in absolute terms (Fig. 2) or relative to Sertoli cell number (not shown). The low-dose ERα agonist (15 μg/kg) was not examined directly because the similar testis weight and histology indicated that the stereological enumeration would be similar. The administration of ERβ agonist (450 μg/kg) had no significant effect on numbers of Sertoli cells or any germ cell population, consistent with overall histology (Fig. 2).

Study 2

Male ERα knockout (αERKO), ERβ knockout (βERKO), or wild-type (WT) mice each on an *hpg* background were treated for 6 wk with either E2 or DHT and compared with untreated littermate controls of the same genotype.

Administration of DHT increased testis, epididymal, and seminal vesicle weights in *hpg*/WT as well as *hpg*/αERKO and *hpg*/βERKO males, whereas E2 increased testis, epididymal, and seminal vesicle weights in *hpg*/WT and *hpg*/βERKO but not *hpg*/αERKO males (Fig. 3). The magnitude of the E2 effects on testis, epididymal, and seminal vesicle weights was markedly less than the DHT effect.

In contrast, DHT had no effect on circulating FSH levels in all of the three genotypes, whereas E2 significantly increased FSH levels in *hpg*/WT and *hpg*/βERKO but not in *hpg*/αERKO mice (Fig. 3). Serum LH levels were not significantly increased by either E2 or DHT in any line (Fig. 3). Serum T was undetectable (<0.1 ng/ml) in all 58 samples including 17 DHT-treated and 20 E2-treated mice. Serum DHT was detectable in all 17 DHT-treated mice (mean 1.67 ± 0.19 ng/ml) with no difference according to ER genotype ($P = 0.64$) and only detectable in one (0.23 ng/ml) of 41 non-DHT-treated mice. Serum E2 was detectable in 11 of 20 estradiol-treated mice (mean 6.4 ± 1.5 pg/ml, median 6.5 pg/ml) with no difference according

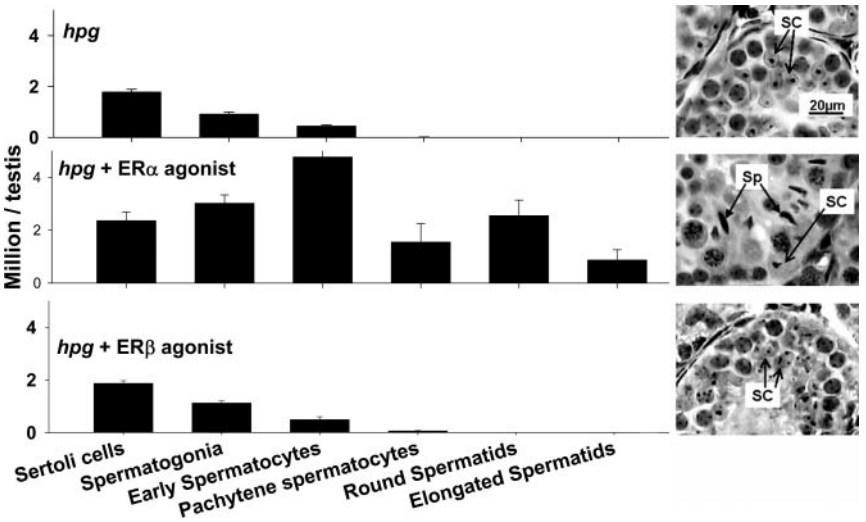

FIG. 2. Sertoli (SC) and germ cell population of the testis in age-matched littermate *hpg* mice that were untreated (*upper panel*) or treated with a selective ERα agonist (16α-LE_2, 45 μg/kg · d for 6 wk; *middle panel*) or a selective ERβ agonist (8β-VE_2, 450 μg/kg · d for 6 wk; *right panel*) for 6 wk. Data expressed as mean and SEM. At the *right* is representative testicular histology from age-matched littermate *hpg* mice that were untreated (*upper panel*) or treated with the selective ERα agonist (*middle panel*) or the selective ERβ agonist (*right panel*). Note elongated spermatids (Sp) present in tubules of *hpg* mice treated with selective ERα but not ERβ agonist. All photomicrographs are at the same magnification. For further details, see text.

to ER genotype ($P = 0.92$) but not detectable (<5 pg/ml) in any of 38 non-E2-treated mice.

Testicular histology in untreated *hpg*/WT, *hpg*/αERKO, and *hpg*/βERKO males had a similar immature morphological appearance (Fig. 4). In both *hpg*/WT and *hpg*/βERKO males, E2 stimulated production of haploid spermatids, although sperm metamorphosis and spermiation appeared limited or incomplete, and tubular diameter was increased but lumina were poorly developed or absent, relative to DHT-induced effects. The E2-treated *hpg*/WT and *hpg*/βERKO testes displayed Sertoli cells with mature morphological characteristics (tripartite nucleoli and basal localization) and Leydig cells with increased vesiculation as compared with untreated *hpg* testis. In contrast, testes of *hpg*/αERKO mice appeared unresponsive to E2 treatment (Fig. 4), whereas DHT treatment induced the equivalent histological completion of spermatogenesis in all three *hpg* genotypes (Fig. 4).

Stereological analysis shows that neither genotype (WT, αERKO, βERKO) nor treatment (E2, DHT) significantly influenced Sertoli cell numbers in this study (Table 1). However, E2 increased all germ cell populations in *hpg*/WT and *hpg*/βERKO groups relative to respective controls but had no stimulatory effect in the *hpg*/αERKO group. In comparison, DHT increased all germ cell populations in each *hpg* genotype and had greater stimulating effect relative to E2, especially on the postmeiotic, haploid germ cell populations (Table 1).

Testicular gene expression

Sertoli cell

In study 1, administration of ERα agonist markedly increased the testicular expression of Sertoli cell-specific genes, *Rhox5* and *Eppin*, compared with expression in untreated *hpg* mice (Fig. 5, *left upper panels*). By contrast, there was little or no increase in *Rhox5* and *Eppin* expression levels in testes from ERβ agonist-treated compared with untreated *hpg* mice (Fig. 5, *left upper panels*).

In study 2, E2 treatment increased the expression levels of *Rhox5* and *Eppin* in *hpg*/*WT* and *hpg*/βERKO but not in *hpg*/αERKO mice, compared with expression levels in untreated controls (Fig. 5, *right upper panels*). By contrast, DHT administration markedly increased expression levels of *Rhox5* and *Eppin* in *hpg* testis, regardless of whether ERβ or ERα was present or absent (Fig.5, *right upper panels*).

Leydig cell

In study 1, treatment with the ERα agonist markedly increased testicular expression of Leydig cell gene markers *Hsd3b1* and *Cyp11a1* compared with untreated *hpg* controls, whereas treatment with the ERβ agonist had no effect (Fig. 5, *left lower panels*).

The effects of ERα agonist on gene expression observed in study 1 were largely replicated by E2 in the testes of *hpg*/WT in study 2 (Fig. 5). The hypogonadal ERβ-null (*hpg*/βERKO) mice were equally responsive to E2, supporting the absence of ERβ agonist effects in study 1. In contrast, all effects of E2 that were observed in *hpg*/WT testes, with the exception of increased *Hsd3b1*, were absent in testes of *hpg*/αERKO mice.

By contrast, DHT treatment had no stimulatory effect on testicular expression of *Cyp11a1* (Fig. 5, *right lower panels*) in *hpg* males, regardless of the presence or absence of ERα or ERβ, whereas *Hsd3b1* expression levels were significantly elevated in DHT-treated *hpg*/αERKO but not *hpg*/WT or *hpg*/βERKO males (Fig. 5, *right lower panels*).

ERβ modulation of DHT effects on nonreproductive organ weights

Administration of DHT increased liver, kidney, and heart, but not spleen weight, in *hpg*/WT as well as *hpg*/αERKO and *hpg*/βERKO males (data not shown). However, the magnitude of the DHT effect on large nonrepro-

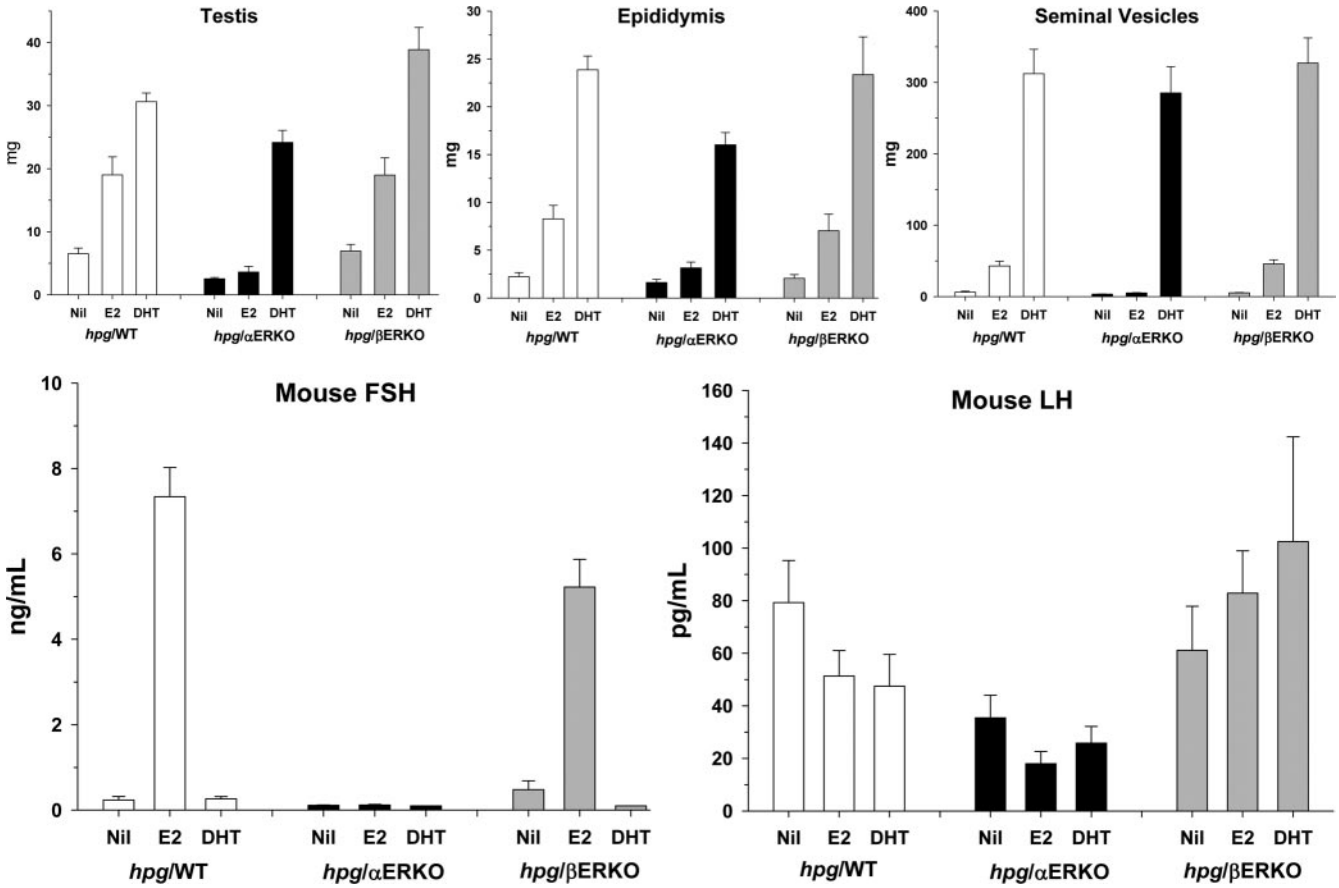

FIG. 3. Testis (*upper left*), epididymis (*upper middle*), and seminal vesicles (*upper right*) together with serum FSH (*lower left*) and LH (*lower right*) in *hpg* mice on wild-type (*hpg*/WT, *white bars*), ERα knockout (*hpg*/αERKO, *black bars*), or ERβ knockout (*hpg*/βERKO, *gray bars*) genetic backgrounds. Data expressed as mean and SEM. For further details, see text.

ductive organ weights (liver, kidney, heart) was markedly greater in the presence of ERβ inactivation (*hpg*/βERKO) compared with *hpg* mice having native (*hpg*/WT) genetic background (Fig. 6). By contrast, the modulating influence of the ERβ gene on DHT effects was less or absent in reproductive organs, in which there was a small but significant increase for the testis and spermatogonia but not other germ cell populations (Table 1 and Fig. 6).

Discussion

Using two complementary pharmacogenetic approaches, the present study shows unequivocally that the E2 induction of spermatogenesis and increased serum FSH levels in *hpg* mice is dependent on an ERα mechanism. Both responses were abolished in mice with an inactivated ERα gene but remained unaffected by inactivation of the ERβ gene. Conversely, both responses were stimulated by a selective ERα agonist but not a selective ERβ agonist. In concert these experiments indicate that ERα activation is involved in both the increased serum FSH and induction of spermatogenesis in *hpg* mice, whereas an ERβ mechanism is involved in neither.

A prominent difference between androgen- and estrogen-mediated induction of spermatogenesis in gonadotropin-deficient *hpg* mice is that only the latter is accompanied by increased serum and pituitary FSH levels (6, 16–18, 30). Given that ERα is generally accepted to be expressed throughout the hypothalamic-pituitary-testicular axis, the concomitant increase in FSH by E2 raises the possibility of both direct and indirect pathways for estrogenic stimulation of spermatogenesis. Several lines of evidence argue against E2 induction of spermatogenesis being direct. These include: 1) the general premise that ERα is largely localized to Leydig and peritubular cells and only weakly in expressed Sertoli cells in mature wild-type and *hpg* testes (36–38) (Supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org); 2) that ERα-mediated effects in Leydig cells are predominantly inhibitory (18, 39–41); 3) that spermatogenesis develops normally in mice null for estrogen synthesis (19) or estrogen action via ERα (20), ERβ (21, 22), or both (23); and 4) that fully fertile germ cells develop in testis in which all somatic cells lack functional ERα (42, 43). To this we add our present observations that the effects of ERα agonist

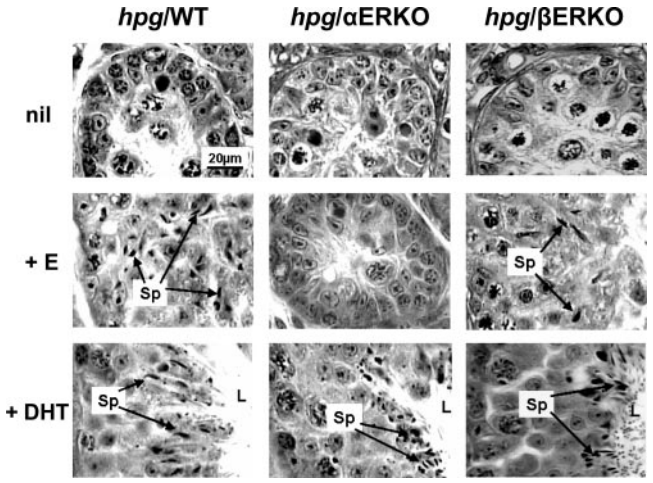

FIG. 4. Testicular histology from age-matched littermate *hpg* mice on different genetic backgrounds comprising WT (*left panels*), αERKO (*middle panels*), and βERKO (*right panels*). The *top row* depicts the untreated, the *middle row* treated with E2, and the *bottom row* treated with DHT for 6 wk. Note elongated spermatids (Sp) indicated by *arrows* present in tubules of *hpg* mice treated with DHT or E2 for the mice on WT and βERKO but not αERKO background. Well-formed seminiferous tubular lumen (indicated by L) is present in all mice treated with DHT but not E2 or untreated. All photomicrographs are at the same magnification. For further details, see text.

alone on postmeiotic germ cell populations are small compared with DHT. Our findings do, however, show prominent effects of E2 via an ERα mechanism on gene expression in Sertoli and Leydig cells, which suggests the possibility for direct effects of E2 via specialized endocrine cells of the testis. It is unlikely that Leydig cells are directly involved in the ERα-mediated E2 induction of spermatogenesis because intratesticular steroid levels decline in E2-treated *hpg* males (18), and concurrent induction of circulating FSH is consistent with elevated Sertoli cell markers and limited postmeiotic germ cell development, typical of known FSH activity in androgen-deficient testes (3, 44). The present findings confirm that E2 administration does not increase serum (16) or testicular (17, 18) levels in *hpg* mice so that the E2-induced increase in seminal vesicle weight must be a direct E2 effect on seminal vesicles as reported previously (45). Therefore, the stimulatory effect of E2 for spermatogenesis in *hpg* mice most likely involves extratesticular ERα actions leading to increased circulating FSH and enhanced Sertoli cell maturation/function.

Nevertheless, given previous reports that AR is required for E2-induced spermatogenesis (17, 18), we must also consider the possibility of ERα-AR heterodimers as a possible direct mechanism for our findings. Actions of an ERα-AR heterodimer has been reported in some breast and prostate tumor cells (46) but not confirmed in other studies (47).

Although the importance of increased FSH to E2-induced spermatogenesis in *hpg* mice is unclear, the current study clearly demonstrates that this effect is ERα dependent. Induction of increased pituitary weight and FSH secretion was observed in ERα agonist-treated *hpg* mice, and the increased circulating FSH was lacking in E2-treated *hpg*/αERKO mice. Because the *hpg* mouse lacks functional GnRH, the E2-induced increase in FSH must be due to effects directly on pituitary gonadotropes and independent of GnRH action. This is consistent with ERα being the predominant ER form expressed in the mouse pituitary (48). Although E2 classically exerts a solely negative feedback effect on the hypothalamic-pituitary axis of mature male mice, the paradoxical E2 stimulation of circulating FSH in *hpg* males resembles the positive estrogen feedback effects mediated by hypothalamic and pituitary

TABLE 1. Stereological enumeration of Sertoli and germ cell populations

Group	n	Sertoli cells	Spermatogonia	Early spermatocytes	Pachytene spermatocytes	Round spermatids	Elongated spermatids
hpg/WT[a]	6	1.60 ± 0.20	0.99 ± 0.11	0.81 ± 0.17	0.09 ± 0.04	0.05 ± 0.05	0.01 ± 0.01
hpg/WT + E2[b]	6	1.77 ± 0.18	1.99 ± 0.15	4.82 ± 0.57	1.03 ± 0.17	0.81 ± 0.19	0.09 ± 0.05
hpg/WT + DHT[c]	5	1.54 ± 0.12	1.74 ± 0.20	4.45 ± 0.24	1.51 ± 0.09	12.2 ± 0.52	11.2 ± 0.47
hpg/αERKO	5	1.39 ± 0.08	0.58 ± 0.07	0.53 ± 0.08	0.06 ± 0.02	0	0
hpg/αERKO + E2[b]	5	1.58 ± 0.08	0.62 ± 0.07	0.26 ± 0.08	0	0	0
hpg/αERKO + DHT[c]	5	1.45 ± 0.16	1.16 ± 0.10	3.18 ± 0.27	0.86 ± 0.18	6.20 ± 0.64	5.58 ± 1.02
hpg/βERKO	6	1.39 ± 0.08	1.20 ± 0.12	2.36 ± 0.42	0.36 ± 0.08	1.19 ± 0.44	0.45 ± 0.26
hpg/βERKO + E2[b]	6	1.79 ± 0.25	2.20 ± 0.34	4.81 ± 0.74	1.20 ± 0.20	4.68 ± 1.95	2.33 ± 1.28
hpg/βERKO + DHT[c]	5	1.47 ± 0.17	2.49 ± 0.30	5.27 ± 0.37	1.77 ± 0.53	14.5 ± 1.50	12.4 ± 1.47
P value							
Genotype[d]		0.50	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment[e]		0.13	<0.001	<0.001	<0.001	<0.001	<0.001
G × T[f]		0.97	0.038	<0.001	0.133	0.002	<0.001

All data from study 2 expressed as mean ± SEM and million cells per testis.

[a] WT means wild-type genetic background with respect to ERα and ERβ; [b] E2 administered by 1 cm subdermal implants filled with recrystallized E2 diluted 1:1000 with cholesterol; [c] DHT administered by 1 cm subdermal implant filled with crystalline DHT; [d] Genotype (*hpg*/WT *vs.* *hpg*/αERKO *vs.* *hpg*/βERKO) main effect from two-way ANOVA; [e] Treatment (untreated *vs.* E2 *vs.* DHT) main effect from two-way ANOVA; [f] Genotype × treatment (G × T) interaction term from two-way ANOVA.

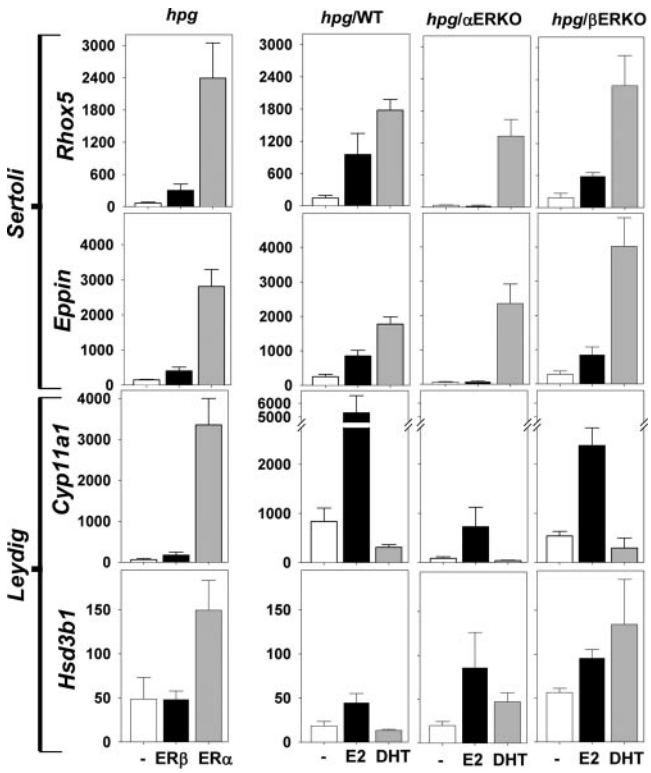

FIG. 5. Total testis *Rhox5*, *Eppin*, *Hsd3b1*, and *Cyp11a1* mRNA expression levels. Data are shown as mean and SEM for treatments (n = 4–10/group) including untreated (−) and ERβ and ERα agonists (*left panel*, study 1) in *hpg* mice and untreated (−), E2, or DHT in *hpg*/WT, *hpg*/αERKO, and *hpg*/βERKO mice (*right three panels*, study 2) along the x-axis. Gene expression levels (y-axis) were measured for two Sertoli cell markers *Rhox5* and *Eppin* (*upper two panels*) and two Leydig cell markers *Cyp11a1* and *Hsd3b1* (*lower two panels*) by real-time qPCR analysis using whole-testis cDNA. The measured mRNA transcript levels are adjusted for *Wbscr1* (housekeeping gene as loading control) and for testis weight (to account for dilution by germ cells) as detailed in text.

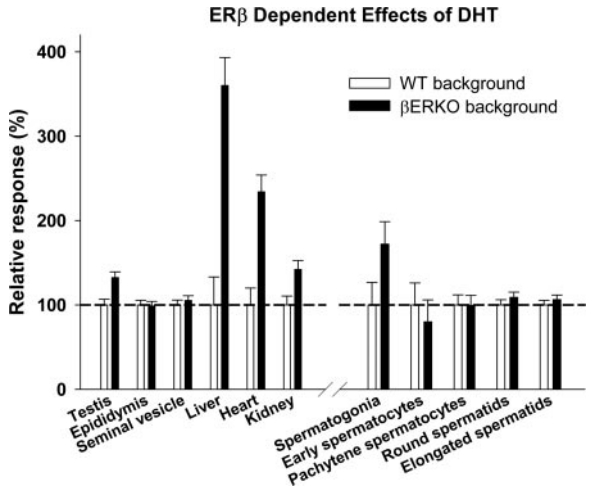

FIG. 6. Magnitude of DHT effect in reproductive and nonreproductive organs in *hpg* mice on βERKO *vs. WT* (*i.e.* functional ERα and ERβ) genetic background. The magnitude of the DHT effect, defined as the increase in organ or tissue weight of the DHT-treated compared with the untreated controls on the same genetic background, with the DHT effect on βERKO background expressed as a percentage of DHT effect on the WT background (set at 100%). Data expressed as mean and SEM. For further details, see text.

mechanisms requiring the pituitary ERα receptor (49). The neuroendocrine mechanism underlying the ovulatory LH surge (with a concomitant FSH surge) characteristic of the mature female reproductive system is usually extinguished in males by neonatal androgen imprinting (50), although such a latent mechanism may still be evoked by toxicological doses of synthetic estrogens (51). Beyond establishing the existence of this pathway, whether it is a physiological mechanism or vestigial pharmacological reflex cannot be resolved by the present study.

This study finds no evidence for ERβ involvement in E2-induced increased serum FSH or spermatogenesis. Genetic loss of ERβ activity on the *hpg* background had no effect on E2-induced FSH levels, testicular growth, and spermatogenesis. Similarly, a selective ERβ agonist failed to stimulate testis, seminal vesicle, prostate, and pituitary growth or to increase Sertoli cell and spermatogonial numbers, unlike the ERα agonist response. Furthermore, loss of ERβ had no effect on DHT-induced spermatogenesis. Our present data suggest that any immunohistochemical evidence of ERβ expression in the testis reflects functional redundancy, as is also the case for ERα expression in germ cells (43). Furthermore, the normal development of spermatogenesis and fertility in mice with genetic inactivation of ERβ (21–23) supports the concept that any essential, nonredundant role for ERβ in postnatal testicular function remains to be established.

The present findings show that E2 actions in *hpg* testis increased testicular expression of *Eppin* and *Rhox5*, known AR-dependent, Sertoli cell-specific genes in the postnatal testis (15, 52, 53). The present study also reveals that up-regulated *Eppin* and *Rhox5* expression involves an ERα mechanism. Prominent stimulation of these Sertoli cell transcripts is reproduced by an ERα but not an ERβ agonist and abolished by genetic inactivation of ERα but not ERβ genes. Considering our findings of ERα immunoreactive expression in Sertoli cells (Supplemental Fig. 1) together with the requirement for AR (17, 18), this ERα-mediated E2 effect may involve direct activation of ERα in Sertoli cells or AR-ERα heterodimers, alone or together with FSH. However, this ERα selectivity was distinct from the DHT effect, which, manifested via AR, is unaffected by the inactivation of either ER, which tends to exclude the possibility of a DHT metabolite with ERα or ERβ agonist activity. The ERα-mediated rise in Sertoli cell expression of *Rhox5* and *Eppin* could be due to the concomitant increases in serum FSH, as exogenous FSH treatment increases *Rhox5* and *Eppin* expression in *hpg* testes (54). However, increased circulating FSH levels alone are not sufficient to complete postmeiotic germ cell development in *hpg* mice (3), consistent with the limited spermatogenic

development induced by the ERα agonist in the present experiments. Neither Sertoli cell marker genes had well-defined ER response elements in their promoters.

The present work also demonstrated complex and distinct steroidal regulation of two Leydig cell genes involved in testicular steroidogenesis, *Cyp11a1* (cytochrome P450 side chain cleavage enzyme) and *Hsd3b1* (3β-hydroxysteroid dehydrogenase type 1). Estradiol increased testicular *Cyp11a1* and *Hsd3b1* expression in *hpg* mice, an effect ablated in the absence of ERα activity. This may reflect direct effects of E2 on Leydig cells because circulating levels of LH, the major Leydig cell trophic hormone, are fully suppressed in *hpg* mice, supported by failure of E2 to increase Leydig cell number (36). Alternatively, E2-mediated stimulation of Leydig cell transcripts may be due to FSH-mediated paracrine signaling between Sertoli and Leydig cells (34). By contrast to E2 stimulatory actions, DHT did not stimulate testicular *Cyp11a1* expression in *hpg*/WT testes, revealing distinct E2 and androgenic regulation of expression of this steroidogenic enzyme. Furthermore, DHT-induced elevation of testicular *Hsd3b1* expression in ERβ-deficient but not control *hpg*/WT males indicates complex regulation of *Hsd3b1* involving AR and ERβ. Therefore, these *in vivo* models have demonstrated distinct and complex involvement of ER pathways, and AR interaction, in the steroidal regulation of Leydig cell *Cyp11a1* and *Hsd3b1* expression. Our findings provide strong evidence for ERβ-mediated effects of DHT, possibly via its 3β-diol metabolite (55), in nonclassical androgen target tissues like the liver, heart, and kidneys, whereas the effects on reproductive tissues are minimal (testis) or absent (accessory reproductive glands), which warrants further investigation.

In conclusion, the present findings indicate that E2 induction of spermatogenesis in the gonadotropin-deficient mouse depends on an ERα, but not an ERβ, mechanism involving increased pituitary secretion of FSH. The overall biological significance of this mechanism in the normal development and maintenance of spermatogenesis requires further exploration.

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