Estrogenic induction of spermatogenesis in the hypogonadal (*hpg*) mouse: role of androgens

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Abstract

Testicular development is arrested in the hypogonadal (*hpg*) mouse due to a congenital deficiency of hypothalamic gonadotropin-releasing hormone synthesis. Previous studies have demonstrated that chronic treatment of these mice with estradiol induces testicular maturation and qualitatively normal spermatogenesis, but it is not known whether these are direct effects via estrogen receptors expressed in the testis, or indirect actions via the pituitary gland. The aim of the current studies was to determine whether the actions of estradiol require the presence of androgens. Sensitive assays revealed that chronic estradiol treatment produced time-dependent increases in pituitary FSH production but no increases in pituitary LH or testicular testosterone content could be detected. As a functional test of androgen dependence, *hpg* mice were treated for 70 days with estradiol plus Casodex (bicalutamide), an androgen receptor antagonist. Casodex treatment markedly attenuated both the estradiol-induced increase in testicular weight and the proliferation of the seminiferous epithelium, as revealed by morphometric analysis. However, it did not affect the estradiol-induced increase in pituitary FSH content, nor did it affect estradiol-induced increases in the weight of the seminal vesicles and epididymides. We conclude that increased FSH production is not sufficient to explain the increase in testicular development induced by estradiol in *hpg* mice; there is a requirement for functional androgen receptors for induction of testicular growth.

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Introduction

Although follicle-stimulating hormone (FSH) and androgens are considered to be the principal regulators of Sertoli cell proliferation and function, and thus of spermatogenesis (Sharpe *et al.* 2003), it is becoming increasingly clear that estrogens also play a role in this process (see O'Donnell *et al.* 2001 for review). The mammalian testis expresses aromatase (Bourguiba *et al.* 2003), produces significant amounts of estradiol (Weniger 1993) and expresses both alpha and beta forms of the estrogen receptor (ER α and ER β) (Fisher *et al.* 1997, Saunders *et al.* 1998, Zhou *et al.* 2002). The progressive impairment of spermatogenesis observed in transgenic mice deficient in aromatase (Robertson *et al.* 1999) or ER α (Eddy *et al.* 1996, Hess *et al.* 1997) is thought to reflect predominantly a deficit in fluid reuptake in the efferent ducts of the testis, but the expression of ER in Leydig and Sertoli cells raises the possibility that estrogens also have actions within the testis itself. ER β immunoreactivity has also been detected

within the germ cell line in the rat and mouse testis (Saunders *et al.* 1998, Zhou *et al.* 2002) and ER α mRNA and immunoreactivity have been reported in human spermatozoa (Durkee *et al.* 1998). However, the demonstration that transgenic germ cells lacking ER α can be transplanted into a host testis depleted of native germ cells, and can then mature to produce viable offspring (Mahato *et al.* 2000), has focused attention on the actions of estradiol on the somatic components of the testis.

We have previously observed that treatment of hypogonadal (*hpg*) mice with low levels of estradiol causes increases in testicular weight that reflect maturation of the seminiferous epithelium and the production of spermatozoa (Ebling *et al.* 2000). The *hpg* mouse is an excellent animal model in which to investigate the mechanism of action of estrogens on spermatogenesis because it has arrested reproductive development without the need for surgical, endocrine, pharmacological or immunological intervention. This mouse line is infertile due to a congenital

deficiency of hypothalamic gonadotropin-releasing hormone (GnRH) synthesis leading to markedly reduced pituitary contents of luteinizing hormone (LH) and FSH and undetectable or barely measurable serum gonadotropin levels (Cattanach *et al.* 1977, Charlton *et al.* 1983). Consequently, the testes fail to undergo normal postnatal development and, at an adult age, *hpg* testicular androgen production is barely detectable (Sheffield & O'Shaughnessy 1989, Scott *et al.* 1990). Treatment of *hpg* mice with androgen alone is sufficient to induce spermatogenesis and this occurs in the absence of detectable FSH concentrations (Singh *et al.* 1995). Conversely, treatment with recombinant human FSH can induce testicular development in *hpg* mice, reflecting increased proliferation of spermatogonia and increased spermatocyte numbers (Singh & Handelsman 1996). However, in the absence of supplementary testosterone treatment, spermatogenesis was arrested at the spermatocyte stage, with little evidence of formation of round spermatids (Singh & Handelsman 1996). In contrast to the limited effects of treatment of *hpg* mice with FSH alone (Singh & Handelsman 1996), our initial studies have shown qualitatively normal spermatogenesis after treatment with estradiol. The overall aim of the current study was to determine whether this might reflect actions of estradiol other than via pituitary release of FSH and, given the evidence for an absolute requirement for androgen in completion of spermatogenesis in the *hpg* mouse (Singh & Handelsman 1996), we specifically investigated whether the actions of estradiol are androgen-dependent.

The main experimental approach was to determine whether concurrent treatment with Casodex (bicalutamide), a peripherally selective androgen receptor antagonist (Furr *et al.* 1987), could block the stimulatory actions of estradiol treatment in *hpg* mice. Casodex has been shown to induce subtle deficits in spermatogenesis in adult rats, for example, a reduction of germ cell numbers during stage VII of spermatogenesis (Chandolia *et al.* 1991a,b). We also determined whether estradiol treatments that stimulate spermatogenesis could increase pituitary LH production, since our initial studies had only investigated effects on FSH release, and whether estradiol treatment could increase intratesticular androgen levels. Our initial studies in *hpg* mice did not detect increases in serum androgen concentrations (Ebling *et al.* 2000) but we predicted that, if the stimulatory action of estradiol was androgen-dependent, then small increases in intratesticular levels of androgen might occur after estradiol treatment. Finally, we determined whether the effects of estradiol could be blocked by concurrent treatment with Faslodex (ICI 182,780), an estrogen receptor antagonist (Wakeling & Bowler 1992). This part of the study was carried out for two reasons: first as a positive control, to confirm that an infrequent administration regime with an antagonist could block the effect of estradiol delivered continuously by subcutaneous silastic implant. Secondly, the stimulatory effects of estradiol on spermatogenesis in *hpg* mice were so unexpected and paradoxical that we

wished to confirm that the action was genuinely via the known genomic estrogen receptors, rather than an interaction of estradiol with other cellular mechanisms.

Materials and Methods

Animals

All studies were conducted using C3H *hpg* mice from a breeding colony at the Biomedical Sciences Unit (BMSU), University of Nottingham, originally derived from a stock procured from Jackson Laboratories, Inc. (Bar Harbor, ME, USA). The mice were aged between 21 and 29 weeks old at the start of the study and were housed with their male littermates. The temperature was maintained between 20 and 25 °C in a 12 h light:12 h darkness photoperiod, with food available *ad libitum*. All animal procedures were approved by the University of Nottingham Local Ethical Review Committee and were carried out in accordance with the Animals Scientific Procedures Act (UK) 1986 (project licence PPL 40/2372). Laboratory Science Animal Association (LASA) guidelines were followed for administration of substances (Morton *et al.* 2001). *hpg* males were identified by their unequivocal phenotype consisting of a micropenis and a shorter anogenital distance. The wild-type littermates are a combination of both homozygous (+/+) and heterozygous (+/*hpg*) genotypes as such mice are phenotypically identical.

Chronic estradiol treatments

Estradiol implants were made by packing 10 mm crystalline 17 β -estradiol (Sigma) mixed 1:50 (2%) by weight with cholesterol into silastic tubing (1.59 mm i.d., 3.18 mm o.d.; Osteotec, Christchurch, Dorset, UK) plugged at either end with silicone medical adhesive (Osteotec). After the adhesive had been allowed to cure for at least 24 h at room temperature, implants were washed with 70% ethanol. They were then incubated in saline for 24 h before implantation, so that release rates would be stabilized by the time the implants were placed under the skin (Ebling *et al.* 2000). A single implant was placed subcutaneously into the flank under general anaesthesia (ketamine 0.4 mg/kg and medetomidine 2 mg/kg, i.p.; Dodge Animal Health, Southampton, UK). Anaesthesia was reversed with 1 mg/kg atipamezole (Antisedan, Pfizer Animal Health, Sandwich, Kent, UK), and mice were treated post-operatively with 5 mg/kg carprofen, a non-steroidal anti-inflammatory analgesic (Rimadyl; Pfizer). These 2% estradiol implants maintain serum estradiol concentrations of approximately 40 pg/ml in adult female or male *hpg* mice, as measured 12 or 35 days after implantation respectively (Ebling *et al.* 2000). Vom Saal *et al.* (1997) reported estradiol levels of 94 pg/ml in fetal male mice, and Bianco *et al.* (2002) reported circulating estradiol concentrations of 14 pg/ml in adult male mice as compared with 19 pg/ml in *hpg* male mice. Thus, the

levels of estradiol produced by 2% estradiol implants are two- to three-fold higher than those found in normal adult male mice, but lower than those found in fetal male mice, and much lower than the 'supraphysiological' serum estradiol levels found in transgenic mice overexpressing human aromatase (~150 pg/ml; Li *et al.* 2001). Moreover, the serum estradiol levels produced by our implants are much lower than those found in rete testis fluid of the adult rat (~250 pg/ml; Free & Jaffe 1979).

Chronic hormone antagonist treatments

Casodex and Faslodex were kindly donated by AstraZeneca Pharmaceuticals (Safety Assessment UK, Alderley Park, Macclesfield, Cheshire, UK). Doses of Faslodex (fulvestrant or ICI 182,780, long-acting intramuscular formulation) were chosen on advice from AstraZeneca Pharmaceuticals. Administration of 250 mg/kg subcutaneously every 30 days to the mouse is known to cause expected anti-estrogenic changes in the male and female reproductive tract. Pharmacokinetic studies in the mouse conducted by AstraZeneca confirm that circulating levels of ICI 182,780 are detected 30 days post dose consistent with a slow release of ICI 182,780 from the long-acting formulation. There is some accumulation on repeat dosing every 30 days. Thus, administration of 250 mg Faslodex/kg every 3 weeks to the *hpg* mouse would be expected to cause estrogen receptor blockade throughout the administration period.

Doses of Casodex (bicalutamide, ICI 176,334) were chosen following consultation with AstraZeneca Pharmaceuticals. Based on studies in mice carried out by AstraZeneca, administration of 5 mg/kg/day orally for 3 months or more is known to cause atrophy of the prostate and seminal vesicles. Administration of 75 mg/kg/day, although well tolerated, causes significant liver hyperplasia associated with hepatic P450 induction. The dose used for the current studies (40 mg/kg three times a week) was chosen as an intermediate effective dose, consistent with extensive dose–response studies in the male rat (Luo *et al.* 1997) and studies in male mice (Skarda 2003).

Experimental designs

Study 1: effect of chronic estradiol treatment on pituitary gonadotropin content

hpg mice received either a 2% estradiol implant or an implant containing cholesterol only. Pituitary glands and testes were collected from subgroups treated with 2% estradiol after 1, 3, 6 and 17 weeks of treatment and from groups treated with cholesterol after 1 and 17 weeks of treatment ($n = 3–5$ per subgroup). Pituitary glands were stored at -20°C , then homogenized in 0.5 ml 0.01 M phosphate-buffered saline (PBS) prior to assay.

*Study 2: pilot study to determine activity of Casodex and Faslodex in *hpg* mice*

Wet uterus weight was recorded in adult female *hpg* mice ($n = 5–7$ per group) that had been treated for 14 days with a silastic capsule containing 100% cholesterol or 2% estradiol in cholesterol, or a single injection of 250 mg/kg Faslodex s.c. or vehicle, or with Casodex in 0.5% Tween 20 solution (oral gavage of 40 mg/kg, three times per week), or with both a 2% estradiol silastic implant and 250 mg/kg Faslodex s.c. Uterine weights were also determined for an age-matched group of wild-type females.

Study 3: effect of androgen-receptor blockade on estradiol-induced testis development

15 *hpg* mice received a 2% estradiol implant and subgroups ($n = 5$ each) were also treated with (i) Casodex in 0.5% Tween 20 solution as an oral gavage at a dose of 40 mg/kg, three times per week; (ii) Faslodex, 250 mg/kg, s.c., at three-week intervals; or (iii) an oral gavage of vehicle (0.5% Tween 20 solution) three times per week. A further group of *hpg* mice received no treatment ($n = 5$), and a final group ($n = 5$) of age-matched wild-type mice also received no treatment. After 70 days of treatment, a blood sample was collected by cardiac puncture under terminal sodium pentobarbitone anesthesia (Rhone Merieux, Harlow, Essex, UK) and mice were then killed by cervical dislocation. Their testes, epididymides and seminal vesicles were excised, trimmed of fat and connective tissue, blotted dry on tissue and weighed. Pituitary glands were removed and stored at -20°C until homogenized in 0.5 ml 0.01 M PBS for assay of gonadotropins. Testes were immersed for up to 24 h in Bouin's fixative, processed through graded alcohols and embedded in paraffin wax blocks. These were serially sectioned at $5\text{ }\mu\text{m}$, the sections mounted upon gelatinized microscope slides and stained with hematoxylin and eosin.

Study 4: effect of chronic estradiol treatment on testicular androgen concentrations

Unfixed testes were collected from *hpg* mice implanted with cholesterol ($n = 7$) or estradiol ($n = 18$) for between 8 and 10 weeks that constituted control groups for other studies, and also from untreated age-matched wild-type males ($n = 3$). Testes were stored at -20°C . Immediately prior to assay, they were homogenized in 0.5 ml 0.01 M phosphate-buffered saline, then extracted twice with 2 ml ether. After the solvent was dried down, the extract was resuspended in 1 ml assay buffer.

Morphometric analyses

The primary morphometric aims were to estimate volumes of seminiferous tubules and seminiferous epithelium within testes. To this end, stereological estimates (Howard & Reed 1998) were made on tissue images randomized for location and orientation by a multistage systematic

random sampling design. For each testis, four sections were randomly chosen for analysis and six fields were selected per section. Fields were sampled as images captured on a Leica DM4000B brightfield microscope via OpenLab software (Improvision, Coventry, West Midlands, UK) and printed to a final magnification of $\times 1000$ with the aid of a stage micrometer scale as an external calibration standard. Volume fractions of testicular component were determined by randomly superimposing a transparent grid comprising 35 test points in a quadratic array. The total number of points landing on a given component (tubule, lumen, epithelium), divided by the total number of points landing on the testis sections, determined the volume fraction. The absolute volume of each component per testis was estimated subsequently from the product of the volume fraction and the processed testicular volume. In order to assess whether changes in tubule volume were attributable to changes in calibre or total length, mean diameters of seminiferous tubules were measured after selecting tubules using an unbiased counting frame (Howard & Reed 1998). In the case of elliptical profiles, the short axis was measured. To give an indication of the degree of germ cell development, each of the 24 fields per testis collected for stereological analysis was also scored by an observer blind to the experimental treatment to indicate the most mature germ cell type present within the field.

Hormone assays

The pituitary gland extracts from study 1 were assayed in duplicate at two or more dilutions using previously validated heterologous radioimmunoassays based upon reagents obtained from the NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases) NIH, Bethesda, MD, USA. The iodination of the tracer preparations (rat FSH, AFP-11454B and rat LH, AFP-11536B) was carried out using the Chloramine-T method. Pituitary extracts and serum samples from wild-type C3H mice diluted in parallel to the standard curve and gonadotropin concentrations were expressed relative to these standards (mouse FSH, AFP-5308D, mouse LH, AFP-5306A). In study 2, FSH and LH contents in pituitary gland extracts were compared using a rat FSH immunoradiometric kit (AH R004, Biocodehycl, Liege, Belgium) and a rat LH RIA kit respectively (AH R002, Biocodehycl). Pituitary extracts from wild-type and *hpg* mice diluted in parallel with the rat FSH standard; the limit of detection was 1.5 ng/ml (7.5 ng/pituitary when 100 μ l extract assayed). All samples were measured in a single assay. The LH assay is a highly sensitive assay (limit of detection 0.12 ng/ml for a 100 μ l sample, i.e. 0.6 ng/pituitary) but, as murine extracts did not dilute in parallel with the rat standards, all experimental samples were compared at the same dilution in a single assay. The values expressed for pituitary content should be considered as relative values rather than absolute values due to this partial cross-reactivity of murine LH with the anti-rat LH antiserum.

Intratesticular androgen was measured using a highly sensitive solid phase ELISA (DRG1559 kit; Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear UK). The limit of detection for a 100 μ l sample of reconstituted extract was 10 pg/tube which equates to 0.1 ng/testis. All samples were extracted in a single batch and assayed in the same ELISA, and are not corrected for the efficiency of the extraction process. This assay is highly specific for testosterone; cross-reactivity with other androgens is less than 0.9%.

Statistical analysis

Data are presented as group mean values $\pm$ S.E. A one-way ANOVA (Statview, Calabas, CA, USA or Prism 2.01, GraphPad Software, San Diego, CA, USA) was used initially to determine whether there were significant overall effects of the various treatments on organ weights, testis morphometry and hormone values. Subsequently, individual groups were compared with a defined control group using Dunnett's procedure for post-hoc comparisons, or multiple comparisons between groups were made using Tukey's post-hoc tests. For histometric studies on the predominant germ cell types in random microscopical fields, numerical frequency distributions were pooled across animals within each group. Distributions in treated and untreated hypogonadal groups were then compared using a 4×3 contingency table design and a chi-squared analysis where 4 is the number of groups and 3 is the number of germ cell types (spermatocytes, round spermatids and elongating spermatids). For statistical purposes, hormone values below the assay limit of detection were assigned the limit value in analyses. *P* values < 0.05 were considered statistically significant.

Results

Study 1: effect of chronic estradiol treatment on pituitary gonadotropin content

Estradiol treatment induced a significant increase in testis weight in *hpg* mice after one week of treatment as compared with untreated or cholesterol-treated control *hpg* mice ($P < 0.05$; Fig. 1, lower panel). Longer durations of estradiol treatment progressively increased testis weight in *hpg* mice ($P < 0.001$). Pituitary FSH content was significantly increased after 3 and 6 weeks of estradiol treatment ($P < 0.05$), and was further increased after 17 weeks of treatment ($P < 0.001$; Fig. 1, upper panel). There was no significant effect of estradiol treatment on pituitary LH content in male *hpg* mice (Fig. 1, middle panel).

Study 2: pilot study to determine activity of Casodex and Faslodex in *hpg* mice

Estradiol treatment for 14 days induced a significant increase in uterine weight in female *hpg* mice ($P < 0.001$) such that the mean uterine weight reached 63% of that in age-matched wild-type females (Fig. 2). The single

Figure 1 Study 1: pituitary content of FSH (top panel) and LH (middle panel), and wet weight of paired testes (bottom panel) in adult *hpg* mice receiving no treatment (open bars), a subcutaneous implant containing cholesterol (Ch; shaded bars) or implants containing 2% estradiol in cholesterol (E; solid bars). Mice were treated for the number of weeks (wk) indicated. Values are group means $\pm$ S.E., $n=3-5$ per group. * $P < 0.05$, *** $P < 0.001$ compared with cholesterol-treated control groups.

treatment of 250 mg/kg Faslodex s.c. was sufficient to attenuate (by 66%) the stimulatory action of the estradiol implant treatment ($P < 0.05$). No estrogen-agonist properties (i.e. stimulatory effects on uterine weight) of Faslodex or Casodex were observed in the female *hpg* mice (Fig. 2).

Study 3: effect of androgen-receptor blockade on estradiol-induced testis and accessory gland development

Estradiol treatment for 10 weeks induced significant increases in the weight of the testes ($P < 0.001$), epididymides ($P < 0.001$) and seminal vesicles ($P < 0.001$) in *hpg* mice (Fig. 3). Testis and epididymal weights after estradiol treatment were five- to six-fold lower than those in age-matched wild-type mice, the weights of the seminal vesicles reached about 10% of those observed in wild-type controls (Fig. 3). Concurrent Casodex treatment significantly and substantially attenuated the estradiol-induced increase in testis weight ($P < 0.001$; Fig. 3, top panel), although testis weights remained above those of

Figure 2 Study 2: wet weight of the uterus in female *hpg* mice that were treated for 14 days with a silastic capsule containing 100% cholesterol (CHOL) or 2% estradiol in cholesterol (E), or a single injection of 250 mg/kg Faslodex s.c. (FASL), or vehicle, or with Casodex (CAS; oral gavage of 40 mg/kg, 3 $\times$ per week), or with both a 2% estradiol silastic implant and 250 mg/kg Faslodex s.c. (E + FASL). Uterine weights were also determined for an age-matched group of wild-type females (+/+). Values are group means $\pm$ S.E., group size as indicated. ^a $P < 0.001$ compared with CHOL and E + FASL groups, ^b $P < 0.05$ compared with CHOL group, ^c $P < 0.001$ compared with all other groups.

the untreated *hpg* group ($P < 0.05$). Casodex treatment did not affect the estradiol-induced increases in the weight of the epididymides or seminal vesicles (Fig. 3, middle and bottom panels). Concurrent Faslodex treatment significantly ($P < 0.001$) attenuated the estradiol-induced increases in the weight of all three organs (Fig. 3).

Morphometric analysis confirmed that the increase in testis weight after estradiol treatment was a reflection of development of the seminiferous epithelium, resulting in significant increases in the volume and diameter of the seminiferous tubules ($P < 0.001$; Table 1, Fig. 4). Results further confirmed that differences in tubule volume were attributable solely to changes in epithelial height (and, hence, tubule diameter) and were not accompanied by changes in total tubule length. Round or elongating spermatids were present in all tubules examined after estradiol treatment (Table 2, Fig. 4). The seminiferous epithelium and total tubule volumes and lengths were nevertheless not as great as in age-matched wild-type mice (Table 1, Fig. 4). Casodex treatment significantly attenuated the effects of estradiol on the seminiferous epithelium ($P < 0.001$), tubule diameter and volume (both $P < 0.01$; Table 1) but did not influence length. Round or elongating spermatids were only observed in a small proportion of tubules (Table 2, Fig. 4), very similar to the tubules in untreated *hpg* mice of a comparable age. Concurrent Faslodex treatment also significantly blocked the estradiol-induced increase in the seminiferous epithelium and total tubule volume (Table 1) and impaired the maturation of germ cells to the spermatid stages (Table 2, Fig. 4).

Figure 3 Study 3: wet weight of the paired testes (top panel), epididymides (middle panel) and seminal vesicles (bottom panel) in adult *hpg* mice receiving no treatment (open bars), or receiving a subcutaneous implant containing 2% estradiol (E) for 10 weeks plus 3 × per week oral gavage of vehicle (VEH; solid bars) or Casodex at 40 mg/kg (CAS; wide-spaced hatched bars), or receiving subcutaneous injections of Faslodex (250 mg/kg) on days 0, 21, 42 and 63 of the trial (FASL; stippled bars). Paired organ weights for an aged-matched group of untreated wild-type C3H mice are depicted on the right (+/+). Values are group means ± S.E., $n=5$ per group. ^a $P < 0.001$ and ^b $P < 0.05$ compared with untreated *hpg* group, ^c $P < 0.001$ compared with estradiol-treated *hpg* + VEH group.

Contingency table analysis of germ cell development in hypogonadal groups (Table 2) confirmed that there were significant between-group differences (chi-squared 263.1 for 6 degrees of freedom, $P < 0.001$). These were

attributable exclusively to estradiol treatment, which reduced the incidence of spermatocytes and increased the incidence of spermatid types in the seminiferous epithelium. Neither concurrent Casodex nor concurrent Faslodex had significant impacts on the distribution of germ cell types compared to untreated *hpg* mice. However, both treatments compromised the enhanced maturation of spermatids seen in estradiol-treated mice.

Estradiol treatment significantly ($P < 0.01$) increased pituitary FSH content (Fig. 5, upper panel). Concurrent Casodex treatment did not affect the estradiol-induced increase in FSH content, whereas Faslodex treatment significantly attenuated the effect of estradiol ($P < 0.05$) (Fig. 5). No significant effects of treatment were observed on pituitary LH content (Fig. 5, lower panel).

Study 4: effect of chronic estradiol treatment on testicular androgen concentrations

Testicular testosterone content was low or below the limit of detection (in 3 of 7 testes) in *hpg* mice treated with cholesterol implants (range 0.10–0.38 ng/testis). Estradiol treatment had no significant effect on mean testicular testosterone content (Fig. 6), 10 of 18 values were below the limit of detection (range 0.10–0.55 ng/testis). The mean testosterone content in testes of *hpg* mice was approximately 0.6% of that in testes of wild-type C3H mice of a similar age (Fig. 6).

Discussion

The current studies confirm and extend our initial observations that estradiol treatment promotes spermatogenesis in *hpg* mice. Using two different immunoassay systems, we have confirmed that this action of estradiol is associated with a progressive increase in pituitary FSH content. This is consistent with the estradiol-induced increases in serum FSH that we previously observed in male *hpg* mice (Ebling *et al.* 2000), and is also consistent with a previous report that estradiol benzoate treatment significantly increased pituitary FSH content and plasma FSH concentrations in adult male *hpg* mice (Stanley *et al.* 1988). In contrast, using two different immunoassay systems, we have not detected any increase in pituitary LH content following estradiol treatment, although there are detectable (but extremely low) levels of immunoreactive LH in the pituitary gland of *hpg* mice. The presence of bioactive LH in the pituitary glands of male *hpg* mice, albeit at far lower levels than in wild-type mice, has long been established (Fink *et al.* 1983). The lack of effect of exogenous estradiol on pituitary LH content in *hpg* mice is also in accordance with the previous study in which estradiol benzoate treatment did not affect pituitary LH content in adult male *hpg* mice (Stanley *et al.* 1988). Consistent with the lack of estradiol-induced increase in pituitary LH content, we failed to observe any increase in testicular testosterone content after estradiol treatment.

Table 1 Morphometric analyses of tests in study 3. Values are group means $\pm$ s.e.

	<i>hpg</i>	<i>hpg</i> + E + VEH	<i>hpg</i> + E + CAS	<i>hpg</i> + E + FASL	+ / +
Number of animals	5	5	5	5	5
Age (days) ^a	205 $\pm$ 1	229 $\pm$ 5	244 $\pm$ 9	244 $\pm$ 12	202 $\pm$ 6
Body weight (g) ^a	36.1 $\pm$ 3.2	27.0 $\pm$ 1.9	26.5 $\pm$ 1.8	26.4 $\pm$ 1.4	31.3 $\pm$ 0.9
Testis weight (mg) ^b	2.5 $\pm$ 0.2	13.7 $\pm$ 1.0*	5.3 $\pm$ 0.3**,†	4.3 $\pm$ 0.7†	85.2 $\pm$ 3.9
Tubule diameter (μ m)	47 $\pm$ 0.6	106 $\pm$ 6.4*	74 $\pm$ 3.5††	62 $\pm$ 2.0†	178 $\pm$ 0.9
Tubule volume (μ l)	1.7 $\pm$ 0.3	12.2 $\pm$ 0.9*	4.1 $\pm$ 0.2††	3.9 $\pm$ 0.7††	70.4 $\pm$ 2.7
Tubule length (m)	1.0 $\pm$ 0.2	1.5 $\pm$ 0.3	1.0 $\pm$ 0.1	1.3 $\pm$ 0.2	2.8 $\pm$ 0.1
Tubule (% testis)	77.1 $\pm$ 1.1	87.5 $\pm$ 2.0**	78.1 $\pm$ 1.8††	85.0 $\pm$ 1.7	81.7 $\pm$ 1.8
Seminiferous epithelium volume (μ l)	1.7 $\pm$ 0.3	9.6 $\pm$ 0.7*	4.1 $\pm$ 0.2†	3.7 $\pm$ 0.6†	52.2 $\pm$ 2.7

^aAge at end of experiment; ^bWet weight.

Superscripts indicate significant differences revealed by Tukey's tests after ANOVA: * P < 0.001 and ** P < 0.05 vs untreated *hpg* group;

† P < 0.001 and †† P < 0.01 vs *hpg* + E + VEH group.

E, estradiol; VEH, vehicle; CAS, casodex; FASL, faslodex; +/+, wild-type males.

Table 2 Germ cell development in study 3. Assessment of most advanced germ cell phenotype in testis sections used for morphometric analyses. Values are expressed as overall percentages, derived from analysis of 120 fields per group (5 mice $\times$ 24 fields per testis).

Most advanced phenotype	<i>hpg</i>	<i>hpg</i> + E + VEH	<i>hpg</i> + E + CAS	<i>hpg</i> + E + FASL	+ / +
Spermatocytes	77%	0%	82%	78%	0%
Round spermatids	13%	44%	8%	5%	0%
Elongating spermatids	10%	56%	11%	17%	100%

Differences between *hpg* groups are significant (chi-squared 236.1 for 6 degrees of freedom, P < 0.001) and focused exclusively on *hpg* + E + VEH mice.

E, estradiol; VEH, vehicle; CAS, casodex; FASL, faslodex; +/+, wild-type males.

Figure 4 Study 3: representative examples of testicular histology. (A) Seminiferous cords in a testis from an untreated *hpg* mouse. (B, C, D) Cross-sections of tubules from *hpg* mice receiving a 2% estradiol implant for 10 weeks and concurrently treated 3 $\times$ per week with an oral gavage of vehicle (B) or Casodex at 40 mg/kg (C), or receiving subcutaneous injections of Faslodex (250 mg/kg) on days 0, 21, 42 and 63 of the trial (D). (E) Seminiferous tubules from an age-matched wild-type litter mate. Note that estradiol treatment (B) stimulates development of the seminiferous epithelium, and that both the androgen receptor antagonist Casodex (C) and the estrogen receptor antagonist Faslodex (D) impair this action of estradiol. The 50 μ m scale bar in panel A (*hpg*) applies to all panels.

Figure 5 Study 3: pituitary FSH (top panel) and LH (bottom panel) content in adult *hpg* mice receiving no treatment (open bars), or receiving a subcutaneous implant containing 2% estradiol (E) for 10 weeks plus 3 $\times$ per week oral gavage of vehicle (VEH; solid bars) or Casodex at 40 mg/kg (CAS; wide-spaced hatched bars), or receiving subcutaneous injections of Faslodex on days 0, 21, 42 and 63 of the trial (FASL; stippled bars). Values are group means $\pm$ S.E., $n=5$ per group. * $P < 0.01$ compared with untreated *hpg* group. Values for aged-matched untreated wild-type C3H mice are depicted on the right (+/+).

Low testosterone concentrations were detectable in the majority of testes analyzed from *hpg* mice, as previously observed (Singh *et al.* 1995). This low level of androgen in the *hpg* testis is clearly of major significance in terms of the action of estradiol because the key finding of the current series of studies is that blockade of the androgen receptor with Casodex greatly attenuated the induction of spermatogenesis by estradiol. However, concurrent Casodex treatment did not block the stimulatory actions of estradiol on pituitary FSH production. The incomplete blockade of the estradiol-induced increase in testis weight could be taken as evidence that some actions of estradiol in the *hpg* male are independent of androgens, or could reflect a sub-maximal dose of the drug in this particular experimental paradigm. Moreover, we cannot fully exclude the possibility that Casodex has some partial agonist activity in *hpg* mice as there were not sufficient mice available to confirm that treatment with Casodex alone produced no effect on the testis. However, the pilot studies in female *hpg* mice did not reveal any estrogen-agonist

Figure 6 Study 4: testicular testosterone content in adult *hpg* mice receiving a subcutaneous implant containing cholesterol (Ch; open bar) or 2% estradiol (E) for 8–10 weeks (solid bar) or in adult wild-type litter mates (+/+; hatched bar). Values are group means $\pm$ S.E., n as indicated.

activity of this compound, and no partial androgen-agonist properties of Casodex were reported in a previous study of its effects in intact and castrated C3H mice (Skarda 2003). We therefore conclude that, in the mouse, testosterone action is critical for the completion of spermatogenesis; FSH alone is not sufficient. Indeed, in a previous study in which *hpg* mice were treated with androgens alone, qualitatively normal spermatogenesis was induced despite an absence of detectable circulating FSH concentrations (Singh *et al.* 1995). Thus, in a qualitative sense, FSH is not even necessary for spermatogenesis in the mouse although, in a quantitative sense, it is extremely important because of its actions on Sertoli cell proliferation and function during the postnatal and pubertal periods (Johnston *et al.* 2004).

The current studies provide several insights into understanding how estradiol interacts with FSH and testosterone to regulate spermatogenesis. We have considered four hypotheses that are illustrated as models in Fig. 7. Models I and II consider actions of estradiol within the testis, since the presence of aromatase in the testis and direct measurements of estradiol production support the view that low levels of estradiol might be produced to act as a paracrine factor (see Carreau 2002 for review). The most direct hypothesis to explain how Casodex blocks the action of estradiol would be that estradiol acts upon Leydig cells to promote androgen production, which then acts upon the seminiferous tubules (Fig. 7, model I). In support of this model is the observation that in both rodents and primates, Leydig cells express ER α (Fisher *et al.* 1997, Pelletier & El-Alfy 2000, Zhou *et al.* 2002), yet there has been no functional explanation for this

Figure 7 Schematic models that may explain estrogenic actions on testis development and function in the *hpg* mouse. Present results favor model IV or a composite model; see text for details. E, estrogen; T, testosterone; AR, androgen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; LH-R, luteinizing hormone receptor; FSH-R, follicle-stimulating hormone receptor.

finding. The limitations of this model are that we were unable to detect any increase in testicular testosterone content after estradiol treatment and are unaware of any other functional evidence that estrogens can promote androgen production by Leydig cells. Indeed, the majority of evidence suggests that estradiol inhibits Leydig cell proliferation and steroidogenesis (see Abney 1999 for review).

A second 'paracrine' hypothesis (Fig. 7, model II) is that estradiol acts upon somatic cells within the seminiferous tubule, either Sertoli cells or perhaps peritubular cells as both have been reported to express ERβ (Saunders *et al.* 1998, van Pelt *et al.* 1999, Zhou *et al.* 2002), and that the actions of estradiol are to augment or synergize with those of testosterone. This model would not require that estradiol treatment increased either pituitary LH production or synthesis of testosterone by Leydig cells in the *hpg* males, as the basal testosterone levels measured may be sufficient to provide a low level of androgenic drive to the tubule with which estradiol can synergize. The major site of estradiol synthesis switches from the Sertoli cell prenatally to the Leydig cell postnatally, so it would seem very plausible that the physiological role of estradiol is as a paracrine signal from the Leydig cell to the tubule. However, when considering how the stimulatory action of estradiol in the *hpg* mouse can be blocked by an anti-androgen, model II is problematic. This is because the postnatal Sertoli cell only appears to express ERβ, but there is no clear evidence for a physiological role of estradiol acting via

ERβ in the testis, as transgenic mice lacking ERβ reportedly show no deficits in fertility (Krege *et al.* 1998).

Models III and IV (Fig. 7) consider actions of estradiol within the pituitary gland, as our Western blot studies reveal the presence of both ERα and ERβ immunoreactivity of appropriate molecular weight in the pituitary gland of male *hpg* mice (Nwagwu *et al.* 2003). Moreover, in female mice, estradiol treatment is known to elicit daily 'ovulatory' surges of LH (model III) and FSH (model IV) – the phenomenon of positive feedback (Murr *et al.* 1973, Bronson & Vom Saal 1979). It might be hypothesized that due to incomplete postnatal masculinization, the male *hpg* mouse can also express such responses to estradiol treatment. Present studies confirm that estradiol treatment of male *hpg* mice increases FSH content, although no significant increase in pituitary LH content was observed after estradiol. Likewise, serum LH concentrations were undetectable in *hpg* mice (E C Furneaux & F J P Ebling, unpublished data). Model III may seem unlikely given the failure to observe an increase in pituitary LH content after estradiol treatment. However, the slight possibility remains that serum LH levels were increased by estradiol but were still below the assay limits of detection.

Model IV (Fig. 7) is supported by a number of lines of evidence. The estradiol-induced rise in FSH has been observed in all studies that we have carried out in *hpg* mice and the model predicts that this increase in FSH acts upon Sertoli cells by synergizing with the low level of testosterone produced by the Leydig cells. Indeed, *in vitro*

studies in which Leydig and Sertoli cells were co-cultured suggest that FSH may augment the production of testosterone by Leydig cells via release of paracrine factors from Sertoli cells (Verhoeven & Cailleau 1990). It should be noted that although the estradiol treatment produced approximately twofold increases in pituitary FSH content in male *hpg* mice, the resulting pituitary FSH content was still an order of magnitude less than that in wild-type male mice (Fig. 5). Circulating FSH concentrations were not measured in the current study, but our previous studies in *hpg* males using similar estradiol treatments revealed that serum FSH concentrations reached 30–40% of those found in wild-type males (Ebling *et al.* 2000). We were unable to detect any increase in testicular testosterone content (study 4) despite these increases in FSH, and a previous study in which *hpg* mice were directly treated with recombinant FSH also failed to detect increases in serum or testicular testosterone (Singh & Handelsman 1996). However, model IV does not require that testosterone levels should increase, merely that a low level of testosterone should be present within the testis that will then permit the actions on the Sertoli cell of the increase in FSH that is induced by estradiol treatment.

Given that multiple cell phenotypes in the reproductive axis express estrogen receptors, one might expect multiple sites of action of estradiol, and thus the physiological effects of estradiol may only be understood via a combination of the proposed models. The common feature of models reflecting the current findings is the critical dependence on testosterone for induction of spermatogenesis; FSH alone cannot support spermatogenesis in mice. Similar conclusions have been reached in other studies in mice where FSH or testosterone signaling has been disrupted by genetic modification. Thus mice lacking production of the FSH beta subunit (Kumar *et al.* 1997) or lacking the FSH receptor (Dierich *et al.* 1998, Abel *et al.* 2000) retain a degree of fertility, whereas those in which the androgen receptor is knocked out are completely infertile (Yeh *et al.* 2002, De Gendt *et al.* 2004). This is true even when the knockout is restricted to Sertoli cells thus allowing other aspects of masculinization to occur (De Gendt *et al.* 2004).

Stimulatory actions of estrogens on testis development have been reported in a number of other rodent models and provide some evidence that estradiol exerts its stimulatory effects via an interaction with FSH. For example, treatment of neonatal rats with estradiol benzoate in the second week of life (postnatal days 5 to 11) stimulates mitosis such that the abundance of type A spermatogonia increases as assessed at day 15 (Kula 1988). In this same experimental paradigm, estradiol benzoate treatment significantly enhanced the actions of FSH on pre-meiotic differentiation at this early stage of development resulting in increased abundance of pachytene spermatocytes (Kula *et al.* 2001). Similar stimulatory effects of diethylstilbestrol treatment in the neonatal period on the initiation of spermatogenesis in rats have also been observed associated

with small increases in circulating FSH (Atanassova *et al.* 2000). Thus, it is possible that estradiol is acting directly on immature pituitary gonadotropes to induce FSH secretion, even though one would normally expect estradiol to act as a negative feedback agent and inhibit FSH production in adult male rodents (Ebling *et al.* 2000). Perhaps the gonadotropes in the pituitary of the *hpg* mouse can be considered to be functionally immature as they have never been exposed to GnRH, hence the pituitary gland in an 'adult' *hpg* mouse responds to estradiol in a similar way to that in the neonatal rat. In support of this conjecture that the gonadotropes of the *hpg* mice retain early developmental characteristics, it has recently been demonstrated that the expression of the melatonin (MT1) receptor is substantially higher in the pituitary gland of the *hpg* mouse than in age-matched litter mates (Johnston *et al.* 2003). A high level of expression of MT1 is a characteristic feature of the fetal-neonatal stage of pituitary gland development in rats and mice (Johnston *et al.* 2003), thus the maintenance of high levels of expression in the pituitary glands of 'adult' *hpg* mice suggests incomplete maturation of this tissue.

The induction of FSH secretion is clearly not the only mechanism by which estradiol promotes testicular function. For example, treatment of adult Siberian hamsters during the seasonal phase of testicular regression with low levels of estradiol can initiate the recrudescence phase, as observed by increased testis weight and histological analysis of germ cell line development (Pak *et al.* 2002). This estrogenic stimulation of seasonal testis reactivation correlates with decreased levels of pituitary and plasma LH and FSH compared with control hamsters treated with cholesterol (Pak *et al.* 2002), implying actions of estradiol within the testis that over-ride the decreased gonadotropin drive. Moreover, Kula *et al.* (2001) demonstrated that combined treatment of neonatal rats with both FSH and estradiol markedly enhanced the efficiency of premeiotic spermatogenesis, suggesting a synergistic effect probably at the level of the Sertoli cell.

The studies in which concurrent treatment with Faslodex blocked the actions of estradiol provide evidence that the stimulatory actions of estrogen are true actions via the known genomic receptors, and not, for example, cross-reactivity of estrogen with androgen receptors, as has been proposed for estrogenic actions on the prostate gland (Yeh *et al.* 1998). Indeed, the concurrent anti-androgen treatment had no effect on estrogen-induced growth of the seminal vesicles and epididymides, whereas concurrent Faslodex treatment completely blocked this. We infer that there are direct actions of estradiol in these tissues, distinct from those mediated via the androgen receptor. Stimulatory actions of estradiol on prostate and seminal vesicle growth in the *hpg* mouse have previously been reported (Bianco *et al.* 2002) and are consistent with the expression of ER α in stromal cells in these tissues (see Bianco *et al.* 2002 for review).

In conclusion, the stimulation of FSH production by estradiol is not sufficient to explain the initiation of spermatogenesis by estradiol in *hpg* mice. Blocking the actions of estradiol by the anti-androgen Casodex demonstrates a requirement for functional androgen receptors for induction of testicular growth. In contrast, the stimulatory actions of estradiol on the seminal vesicles and epididymides, classically considered androgen-responsive organs, are likely to be direct effects via estrogen receptors expressed in those tissues. Testicular maturation is arrested at a pseudo-neonatal stage in the *hpg* mouse due to the chronic gonadotropin insufficiency, so the physiological relevance of these experimental observations is likely to be that estradiol is important in the initial development of the male reproductive axis.

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