Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors

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Abstract

FSH and androgen act to stimulate and maintain spermatogenesis. FSH acts directly on the Sertoli cells to stimulate germ cell number and acts indirectly to increase androgen production by the Leydig cells. In order to differentiate between the direct effects of FSH on spermatogenesis and those mediated indirectly through androgen action, we have crossed hypogonadal (hpg) mice, which lack gonadotrophins, with mice lacking androgen receptors (AR) either ubiquitously (ARKO) or specifically on the Sertoli cells (SCARKO). These hpg.ARKO and hpg.SCARKO mice were treated with recombinant FSH for 7 days and testicular morphology and cell numbers were assessed. In untreated hpg and hpg.SCARKO mice, germ cell development was limited and did not progress beyond the pachytene stage. In hpg.ARKO mice, testes were smaller with fewer Sertoli cells and germ cells compared to hpg mice. Treatment with FSH had no effect on Sertoli cell number but significantly increased germ cell numbers in all groups. In hpg mice, FSH increased the numbers of spermatogonia and spermatocytes, and induced round spermatid formation. In hpg.SCARKO and hpg.ARKO mice, in contrast, only spermatogonial and spermatocyte numbers were increased with no formation of spermatids. Leydig cell numbers were increased by FSH in hpg and hpg.SCARKO mice but not in hpg.ARKO mice. Results show that in rodents 1) FSH acts to stimulate spermatogenesis through an increase in spermatogonial number and subsequent entry of these cells into meiosis, 2) FSH has no direct effect on the completion of meiosis and 3) FSH effects on Leydig cell number are mediated through interstitial ARs.

Introduction

Sertoli cell function and spermatogenesis are dependent upon the actions of FSH and androgen. This is clearly seen in hypogonadal (hpg) mice, which lack circulating FSH and LH and are infertile with associated failure of the germ cells to progress beyond early meiosis (Cattanach et al. 1977). Treatment of hpg mice with FSH stimulates germ cell proliferation, with an increase in spermatogonial and spermatocyte numbers, and induces spermatid formation (Singh & Handelsman 1996). This is similar to the effects of FSH in the GnRH-immunised or hypophysectomised rat (Vihko et al. 1991, Russell et al. 1993, McLachlan et al. 1995), and in tandem with studies on FSH receptor knockout (FSHRKO) mice, our current understanding is that the primary function of FSH is to maintain germ cell numbers and to promote germ cell progression through meiosis (Dierich et al. 1998). One major problem with the study of FSH action in these models, however, is dissociating the effects of FSH from those of androgen. In early studies, the FSH preparations used contained low levels of LH, which could induce androgen production by the Leydig cells. Use of recombinant FSH has overcome this problem but there remains the issue that FSH has been shown to induce Leydig cell function, probably indirectly through stimulation of the Sertoli cells (Johnson & Ewing 1971, Chen et al. 1976, Vihko et al. 1991). We have shown that FSH will increase androgen levels in the hpg testis and that it will induce expression of androgen-dependent Sertoli cell genes such as Rhox5 (Abel et al. 2009). It remains likely, therefore, that some of the effects of FSH on spermatogenesis, seen in models such as the hpg or hypophysectomised animal, are mediated indirectly through stimulation of androgen production. To investigate the role of androgen in mediating FSH action and to identify the direct effects of FSH, we have generated hpg mice...
lacking androgen receptors (AR) either ubiquitously (hpg.ARKO) or specifically on the Sertoli cells (hpg.SCARKO). Treatment of these mice with FSH allows us to dissect the direct effects of FSH on testicular function from those mediated by androgen action through the Sertoli cell or other androgen-responsive cells in the testis.

Results

Testis volume, seminal vesicle weight and testosterone levels

Testicular volume was similar in hpg and hpg.SCARKO mice but was significantly reduced in hpg.ARKO mice (Fig. 1). Treatment of the animals with FSH for 7 days increased testis volume significantly in all three groups. Seminal vesicle weights were similar in hpg and hpg.SCARKO mice, and were significantly increased by FSH (Table 1). The hpg.ARKO mice do not develop seminal vesicles. Intratesticular testosterone levels were significantly increased in hpg mice after 7 days treatment with FSH but were unaffected in hpg.SCARKO or hpg.ARKO mice (Fig. 1). For comparison, intratesticular testosterone levels in normal adult mice are about 50 pmol/testis (Baker et al. 2003, O’Shaughnessy et al. 2008).

Morphology

As described previously, and with the variation noted in Materials and Methods, spermatogenesis in untreated hpg mice was severely disrupted with spermatocyte progression only as far as the pachytene stage and no spermatids present (Fig. 2). Few Leydig cells were apparent within the interstitial space (Fig. 2). Testes from untreated hpg.SCARKO mice had a similar morphology with spermatogenesis progressing to the primary spermatocyte stage (Fig. 2). In hpg.ARKO mice, the tubules were smaller, and while spermatogenesis progressed to the same stage as the hpg mice, fewer spermatocytes were apparent. Crystalline structures with the appearance of microliths were present in some tubules, as previously reported (O’Shaughnessy et al. 2009).

Treatment of hpg mice with FSH caused an increase in seminiferous tubule diameter (Figs 2 and 3), clear establishment of a tubular lumen and an increase in germ cell number (Fig. 2). In some tubules, spermatogenesis progressed to the round spermatid stage (Fig. 2). In hpg.SCARKO and hpg.ARKO mice, there was also an increase in tubule diameter, although not as marked as in the hpg mice after FSH (Figs 2 and 3). And while there was a clear increase in germ cell number, there was no apparent progression beyond the primary spermatocyte stage.

FSH treatment caused an apparent increase in interstitial space and cell numbers in hpg and hpg.SCARKO mice (Fig. 2). There was no clear effect of FSH on the interstitium of the hpg.ARKO mouse (Fig. 2).

Stereology

Sertoli cell number was similar in hpg and hpg.SCARKO mice but was significantly reduced in hpg.ARKO mice (Fig. 3). Treatment with FSH had no significant effect on Sertoli cell number in any group. Leydig cell number was similar in hpg and hpg.SCARKO mice but was slightly reduced in hpg.ARKO mice compared with the hpg.SCARKO (Fig. 3). Treatment with FSH increased Leydig cell number in hpg and hpg.SCARKO mice but had no effect in hpg.ARKO mice.
Spermatogonial, spermatocyte and total germ cell numbers were similar in hpg and hpg.SCARKO mice but were significantly reduced in hpg.ARKO mice (Fig. 4). Treatment with FSH increased total germ cell number in all three groups by three- to fourfold (Fig. 4). Statistical analysis showed no interaction between the effects of FSH and animal phenotype, indicating that the effect of FSH was similar in all three groups. Further analysis of germ cell types showed that spermatogonial and spermatocyte numbers were increased by FSH in all groups with no significant interaction. As indicated above, FSH treatment induced development of round spermatids only in hpg mice and not in hpg.SCARKO or hpg.ARKO mice.

Discussion

The function that FSH plays in the regulation of spermatogenesis in rodents and higher mammals has been the subject of considerable study, and continuing uncertainty, since the early pioneering work in the 1930s, which showed that FSH could partially restore spermatogenesis in rats after hypophysectomy (Greep et al. 1936). Problems with LH contamination of FSH preparations limited the progress for a number of years, but a major development in our understanding of FSH action in the rodent came with the generation of mice lacking FSH (FSHbKO) or the FSH receptor (FSHRKO; Kumar et al. 1997, Abel et al. 2000, Krishnamurthy et al. 2000). These animals were fertile, but they showed that FSH was required for normal development of Sertoli cell and germ cell numbers (Kumar et al. 1997, Abel et al. 2000, Krishnamurthy et al. 2000). In addition, more recent study of mice lacking both FSHR and AR on the Sertoli cells showed that FSH acts to increase the number of spermatogonia and the entry of these cells into meiosis (Abel et al. 2008). From these studies, it was clear that FSH was required for normal testicular development, but it still remained uncertain how many of the effects of FSH were mediated directly through FSH action and how many were dependent on indirect alteration of androgen levels. This study was designed, therefore, to determine which effects of FSH on testicular function are direct, which are dependent on androgen action and whether those effects of androgen are mediated through the Sertoli cell.

Sertoli cell numbers in the mouse are normally determined by around postnatal day 15. Factors regulating Sertoli cell number are not fully understood but androgens, probably acting through the peritubular myoid cells (PMCs), stimulate proliferation in utero, while FSH is required post-natally (Johnston et al. 2004, Tan et al. 2005). In the adult hpg mouse, Sertoli cell numbers are about 50% of normal (Baker & O’Shaughnessy 2001, Haywood et al. 2003), reflecting the loss of both FSH and androgen post-natally in these mice. Interestingly, there was a reduction in Sertoli cell numbers in the hpg.ARKO mice compared with the hpg.ARKO mice (insets D and F). Representative Sertoli cells and spermatocytes are indicated by black and white arrows respectively. Photomicrographs are from semi-thin sections of testes fixed in paraformaldehyde/gluteraldehyde apart from the insets, which are from Bouin’s-fixed testes. The bar represents 20 μm.
hpg.ARKO mice are likely to be due to androgen action in the foetal testis. The number of Sertoli cells in hpg.SCARKO mice was the same as in the hpg, consistent with data showing that SCARKO mice have a normal contingent of Sertoli cells (De Gendt et al. 2004, Abel et al. 2008). This provides further confirmation that androgen effects on Sertoli cell numbers are independent of direct androgen action on the Sertoli cell (Johnston et al. 2004, Tan et al. 2005). Failure of FSH to affect Sertoli cell number in any group in this study is consistent with earlier findings (O’Shaughnessy et al. 1992, Singh & Handelsman 1996) and indicates that the Sertoli cells in the adult hpg are no longer sensitive to the mitogenic effects of FSH.

Differences in germ cell numbers between untreated hpg and hpg.ARKO mice could be due to the presence of very low levels of androgen in the post-natal hpg testis or, as above, to the effects of androgen action in utero. Androgen action in utero appears more likely since the presence of endogenous testicular androgen post-natally would probably lead to a difference in germ cell number between hpg and hpg.SCARKO mice, as androgen action through the Sertoli cell is clearly required for normal germ cell development (De Gendt et al. 2004). If the effects are due to androgen action in utero, differences between hpg and hpg.SCARKO mice would not arise since Sertoli cells do not express ARs until after birth (Bremner et al. 1994, Zhou et al. 1996). In the foetal testis, ARs are expressed predominantly on PMCs, which would suggest that the differences in germ cell number between adult hpg and hpg.ARKO mice are due to androgen action through the PMCs in utero. Interestingly, it has recently been shown that androgen action through the PMCs is essential post-natally for the development of normal spermatogenesis (Welsh et al. 2009). It has been reported that primordial germ cells express the AR, which would offer an alternative mode of action of androgens in utero (Merlet et al. 2007). The direct effect of androgen on the germ cells is reported to be inhibitory, however (Merlet et al. 2007), suggesting that this is unlikely to explain differences between hpg and hpg.ARKO mice.

Figure 3  Effect of FSH on (A) tubule diameter, (B) Sertoli cell number and (C) Leydig cell number in hpg, hpg.SCARKO and hpg.ARKO mice. An interaction indicates that the effect of FSH was significantly different in the three animal groups. In untreated animals, groups with different letter superscripts were significantly (*P<0.05) different. If FSH had a significant effect on a particular animal group, this is indicated by *. Mean ± S.E.M. is shown. Animal numbers, hpg n = 6; hpg+FSH n = 3; hpg.SCARKO n = 4; hpg.SCARKO+FSH n = 3; hpg.ARKO n = 3; hpg.ARKO+FSH n = 3.

Figure 4  Effect of FSH on numbers of (A) total germ cell, (B) spermatogonia, (C) spermatocytes and (D) round spermatids per testis in hpg, hpg.SCARKO and hpg.ARKO mice. In untreated animals, groups with different letter superscripts were significantly different (*P<0.05). If FSH had a significant effect on a particular animal group, this is indicated by *. Mean ± S.E.M. is shown. Animal numbers, hpg n = 6; hpg+FSH n = 3; hpg.SCARKO n = 4; hpg.SCARKO+FSH n = 3; hpg.ARKO n = 3; hpg.ARKO+FSH n = 3.
A number of previous studies, using a variety of different models including the hpg mouse, hpg mouse expressing FSH, GNRH-immunised rat and hypophysectomised rat, have reported that FSH acts to increase the numbers of spermatogonia, spermatocytes and round spermatids (Vihko et al. 1991, Bremner et al. 1995, McLachlan et al. 1995, Russell et al. 1998, Haywood et al. 2003). In the hpg, hpg,SCARKO and hpg,AR KO models, FSH increased the total germ cell number and spermatogonial and spermatocyte numbers, consistent with earlier studies, and showed that these effects of FSH are independent of androgen action through the Sertoli cell or any other androgen-responsive cell in the testis. FSH treatment also stimulated round spermatid formation in the hpg testis, as previously reported (Singh & Handelsman 1996, Haywood et al. 2003), although spermatid numbers were only about 5% of spermatocyte numbers. In contrast, FSH failed to stimulate the generation of round spermatids in the hpg,SCARKO and hpg,AR KO mice showing that this effect of FSH is entirely dependent on androgen action through the Sertoli cells. This is consistent with earlier studies using hypophysectomised rats, which showed that stimulation of post-meiotic germ cell formation by FSH was partially inhibited by the AR antagonist flutamide (Russell et al. 1998) or ethane dimethane sulphonate, which acts to destroy Leydig cells (Matikainen et al. 1994). One caveat to these studies is that the hpg mice used here will have developed in a gonadotrophin-free environment and may not, therefore, show the same response to FSH as the normal adult animal. The consistency between results using the hpg models and other data described above using different animal models would suggest, however, that these results are relevant to normal spermatogenesis. Overall, therefore, the results from this and earlier studies show that, in rodents FSH acts to stimulate spermatogenesis through an increase in spermatogonial number and subsequent entry of these cells into meiosis. Completion of meiosis appears to be absolutely dependent on the action of androgen.

Generally, insofar as it has been studied, the effects of FSH appear to be similar across different mammalian species. In rhesus and cynomolgus monkeys, FSH appears to act primarily to increase the number of spermatogonia (Marshall et al. 1986, 1995, Simorangkir et al. 2009), while in sheep immunisation against FSH reduces spermatogonial numbers (Kilgour et al. 1998). The role of FSH in human spermatogenesis remains somewhat unclear since there is a conflict between the effects of FSHβ deletion and FSHR deletion (Tapanainen et al. 1997, Lindstedt et al. 1998, Phillip et al. 1998, Layman et al. 2002) and because treatment of infertile hypogonadotropic men is based on treatment with hCG making it difficult to establish effects of FSH. Nevertheless, the prevailing evidence suggests that data from rodents are relevant generally and that the primary effect of FSH is to maintain spermatogenesis quantitatively through effects on spermatogonial numbers.

Numerous studies have shown that FSH will stimulate Leydig cell function through an indirect mechanism, which is assumed to involve release of paracrine factors from the Sertoli cells following direct stimulation of the FSH receptor (Chen et al. 1976, Vihko et al. 1991). In this study, intratesticular testosterone levels were only increased by FSH in the hpg group and not the hpg,SCARKO or hpg,AR KO groups. This contrasts with the increase in seminal vesicle weights after FSH treatment in both hpg and hpg,SCARKO groups suggesting that there is an increase in testosterone in the hpg,SCARKO at the start of treatment, but that this is not maintained up to 7 days. Baines et al. (2008) have shown previously that FSH will increase Leydig cell number in the adult hpg. Our results confirm this observation and show that the effects of FSH on Leydig cell number in the hpg mouse are mediated through androgen action not involving the Sertoli cells. Since Leydig cells express ARs (Zhou et al. 2002), the simplest explanation is that FSH indirectly stimulates androgen production by the Leydig cells, which, in turn, acts directly on the Leydig cells to induce proliferation or, possibly, differentiation from precursor stem cells. This is consistent with earlier data showing that Leydig cell number is reduced in Tim and AR KO mice (O’Shaughnessy et al. 2002, De Gendt et al. 2005).

In conclusion, the design of this study has allowed us to dissect the direct effects of FSH away from those of androgen and to show that FSH acts only during the initial stages of spermatogenesis to optimise germ cell number. Results also demonstrate that FSH cannot stimulate completion of meiosis, which is entirely dependent on androgen action.

Materials and Methods

Animals and treatments

All mice were bred and all procedures carried out under UK Home Office Licence and with the approval of a local ethical review committee. SCARKO and AR KO mice have been previously generated by crossing female mice carrying an Ar with a floxed exon 2 (Arf) with male mice expressing Cre under the regulation of the Sertoli cell-specific promoter Anh or the ubiquitous promoter Pkg1 (Lecureuil et al. 2002, De Gendt et al. 2004). In order to produce hpg,SCARKO mice, hpg mice (C3H/HeH-101/H) were initially crossed with mice carrying the Arf allele (Swiss-Webster/129) and with mice carrying the Anh-Cre transgene (C57-BL6/J). From these crosses, female mice heterozygous for the GNRH deletion (hpgf/+) and homozygous for the Arf allele were crossed with hpgf/+Anh-Cre males (heterozygous or homozygous for Cre) to generate hpg,SCARKO mice. The hpg deletion and Arf allele were detected by PCR analysis of ear clip lysates (Lang 1991) and excision of the floxed Ar confirmed at termination by PCR of testicular DNA (De Gendt et al. 2004). The generation of
hpghARKO mice was similar except that Pgk-Cre (C57-BL6/SJL) replaced Amh-Cre. The hpghARKO males were detected by PCR of ear clip lysates for Sry and deletion of GNRH and confirmed at termination by the absence of epididymides, seminal vesicles and ductus deferens. The hpgh mice used in this study were generated from the same litters producing hpghSCARKO and hpghARKO mice.

To determine the effects of FSH treatment, adult (10 weeks of age) male hpgh, hpghSCARKO and hpghARKO mice were injected s.c. with 8 IU recombinant human FSH (Serono Ltd) in 0.2 ml PBS (pH 7.4, Sigma–Aldrich) once daily for 7 days. The manufacturer’s datasheet states that the hormone preparation contains no LH activity. The dose used was based on preliminary dose–response studies showing that 8 IU/day caused a maximum increase in testis weight over a 1-week period. Mice were killed on day 8 (24 h after the last injection), and testes were snap frozen in liquid nitrogen or fixed overnight. Fixation was either in Bouin’s for subsequent morphometric analysis or 4% paraformaldehyde/1% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for preparation of semi-thin sections.

Testicular morphology in the hpgh mouse has been described previously in a number of publications (Cattanach et al. 1977, Singh & Handelsman 1996, Myers et al. 2005, Lim et al. 2008). Spermatogenesis can progress to the pachytene spermatocyte stage in the hpgh mouse, and numbers of spermatocytes and spermatogonia are similar (Singh & Handelsman 1996, Myers et al. 2005, Lim et al. 2008). In the hpgh mice produced for this study, ~80% were of this phenotype but the remaining 20% of animals had <5% of the expected number of spermatocytes present. All the mice used in this study are generated by crossing mouse lines that are on different backgrounds, and it appears likely that the altered phenotype in some animals is caused by background effects. Mice with a clear, marked reduction in spermatocyte numbers were not used in the study reported here.

**Hormone measurements**

Intratesticular levels of testosterone were measured by RIA following ethanol extraction, as previously described (O’Shaughnessy & Sheffield 1990). The limit of detection of the assay was 40 fmol/ml, which equates to 20 fmol/testis after extraction. The intra- and inter-assay coefficients of variation were 6.8 and 12.1% respectively. Cross reactivity with androstenedione and 5α-androstane-3α,17β-diol was 3.0 and 8.1% respectively.

**Histology and stereology**

To prepare semi-thin (1 μm) sections, testes were embedded in araldite and sections were stained with toluidine blue. For stereological analysis, testes were embedded in Technovit 7100 resin, cut into sections (20 μm) and stained with Harris’s haematoxylin. The total testis volume was estimated using the Cavalieri principle (Mayhew 1992). The optical dissector technique (Wreford 1995) was used to count the number of Sertoli cells, germ cells and Leydig cells in each testis. Each cell type was identified by previously described criteria (Russell et al. 1990, Baker & O’Shaughnessy 2001). The numerical density of each cell type was estimated using an Olympus BX50 microscope fitted with a motorised stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA, USA). Tubule diameter was measured directly in a total of at least 36 tubules from three sections.

**Statistical analysis**

Most data sets were analysed using two-factor ANOVA with effects of FSH and AR deletion as the factors. Where the interaction between factors was significant, this indicates that the effect of FSH was altered by deletion of the AR. To determine whether differences between individual groups were significant, t-tests were employed using the pooled variance from the ANOVA. Data were log transformed where appropriate to avoid heterogeneity of variance. Data on intratesticular testosterone were analysed by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney test.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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