Testicular steroidogenesis in the testicular feminized (Tfm) mouse: loss of 17α-hydroxylase activity

L. Murphy and P. J. O'Shaughnessy
Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London

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ABSTRACT

Testicular feminized (Tfm) mice are totally insensitive to androgen and may be used to study the role of the androgen receptor in normal development and function. We have examined testicular and Leydig cell steroidogenesis in Tfm mice. Serum bioactive LH was high in Tfm mice but serum testosterone was low and this was associated with a severe reduction in testicular testosterone production in vitro. Examination of [3H]pregnenolone metabolism by testes of Tfm mice indicated that progesterone, rather than testosterone, was the major steroid produced. Leydig cells were isolated from normal and Tfm mice and from normal mice in which testicular descent was surgically prevented before puberty. As in whole testes, androgen production in response to human chorionic gonadotrophin was severely reduced in Leydig cells from testes of Tfm mice compared with normal or cryptorchid groups. In contrast, progesterone production by Leydig cells from testes of Tfm mice was markedly increased in comparison with other groups. Total steroid production (progesterone plus androstenedione plus testosterone), however, was only 24% of normal in Leydig cells from Tfm mice. The pattern of steroid production by Leydig cells from cryptorchid testes was similar to control, although total steroid production was reduced to about 50% (this was significantly higher than the Tfm group, P<0.05). The high progesterone/androgen ratio in testes from Tfm mice suggested that 17α-hydroxylase was depleted in these animals. To confirm this, activity of the four major steroidalogenic enzymes associated with the smooth endoplasmic reticulum was measured. Activities (per testis) of 3β-hydroxysteroid dehydrogenase and 5α-reductase were normal in Tfm and cryptorchid mice but, as expected, 17α-hydroxylase activity was only 2.4% of control and 4.5% of cryptorchid testes. 17-Ketosteroid reductase activity was markedly reduced in cryptorchid testes (14.4% of control) but there was a further reduction in testes from Tfm mice to 0.1% of control. Results show that the Tfm mutation is associated with marked loss of 17α-hydroxylase and 17-ketosteroid reductase activities. This suggests that these enzymes may require receptor-mediated androgen stimulation during development to express normal activity. Journal of Endocrinology (1991) 131, 443-449

INTRODUCTION

Androgen insensitivity, also known as testicular feminization, is an X-linked disorder which arises from a lack of tissue responsiveness to androgen. In rats and mice, XY testicular feminized (Tfm) animals have an external female phenotype with small abdominal testes and no mesonephric or paramesonephric duct derivatives (Bardin, Bullock, Schneider et al. 1970; Lyon & Hawkes, 1970; Goldstein & Wilson, 1972). The Tfm rat has a very low sensitivity to androgens caused by a single base mutation in the androgen receptor gene which reduces androgen-binding capacity to 10-15% of normal (Yarbrough, Quarmby, Simental et al. 1990). In contrast, the Tfm mouse is completely insensitive to androgens (Ohno, 1971; Bardin, Bullock, Sherins et al. 1973) due to a single base deletion in the gene encoding the receptor (Charest, Zhou, Lubahn et al. 1991). This deletion causes a frameshift mutation which results in premature termination of translation and production of a shortened androgen receptor which should lack both DNA- and steroid-binding domains (Charest et al. 1991). The Tfm mouse offers, therefore, an ideal model with which to examine the role of androgen receptor interactions in normal development and function.

Androgens have been shown to have direct, receptor-mediated, effects on both the Leydig cells and Sertoli cells of the testis (Louis & Fritz, 1979; Adashi & Hsueh, 1981; Verhoeven & Cailleau, 1988). In normal Leydig cells, androgens act to inhibit steroidogenesis

by decreasing synthesis of 17α-hydroxylase (Hales, Sha & Payne, 1987) and this may be a normal homeostatic mechanism within the Leydig cell. Hypogonadal (hpg) mice lack gonadotrophins and their testes do not develop postnatally (Cattanach, Iddon, Charlton et al. 1977). Treatment of these animals with testosterone has no effect on Leydig cell androgen production (O'Shaughnessy & Sheffield, 1990), suggesting that testosterone alone has no trophic effect on Leydig cell function. It is possible, however, that the action of androgen, through its receptor, is required for normal Leydig cell development, and there is evidence from previous studies (Schneider & Bardin, 1970; Goldstein & Wilson, 1972; Blackburn, Chung, Bullock & Bardin, 1973; Goldman & Klingele, 1974) that steroidogenesis is abnormal in Tfm rats and mice. In this study we have examined testicular steroidogenesis in the Tfm mouse and we show that this mutation is associated with loss of activity of the steroidogenic enzymes 17α-hydroxylase and 17-ketosteroid reductase.

**MATERIALS AND METHODS**

**Materials**

[4,7-3H]Pregnenolone, [1,2,6,7-3H]progesterone, [1,2,6,7-3H]androstenedione, [1,2,6,7-3H]testosterone and 14C-labelled steroids to measure recovery were purchased from Amersham International plc (Amersham, Bucks, U.K.) and were purified by thin-layer chromatography (TLC) before use. Non-radioactive steroids were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or Steraloids Ltd (Croydon, Surrey, U.K.). Organic solvents were purchased from BDH (Poole, Dorset, U.K.) while other chemicals and reagents, unless stated, were from Sigma Chemical Co.

**Animals**

Adult Tfm/Y mice and normal adults of the same strain (C3H/HeH and 101/H; Cattanach et al. 1977) were obtained from the MRC Radiobiology Unit, Harwell, Berks, U.K. Normal animals were also bred at the Royal Veterinary College.

Testes of Tfm mice fail to descend and remain intraabdominal in the adult animal. It was necessary, therefore, to examine the effect of this intraabdominal location on testicular steroidogenesis. Normal animals were rendered bilaterally cryptorchid when aged 19 days since this is before the time of final descent in the normal mouse. The operations were as described previously (O'Shaughnessy & Sheffield, 1991) except that both testes were moved into the abdomen and the testes were attached to the body wall by a single suture through the proper ligament of the testis. All animals were subsequently used for experiments when aged 3–4 months. Animals were killed by decapitation, blood was collected and testes were quickly removed for freezing or tissue incubation.

**Tissue incubations**

**Whole testes**

Testes were decapsulated and incubated in 2 ml Medium 199 (M199) (Gibco, Paisley, Strathclyde, U.K.) containing 0.1% bovine serum albumin (pH 7.4). Incubations were for 4 h at 32 °C under 5% CO2 in air. One testis from each animal was incubated in medium alone (basal) while the contralateral testis was incubated in the presence of a saturating concentration of human chorionic gonadotrophin (hCG; 200 mIU/ml). Medium from each incubation was frozen until assayed for testosterone content by radioimmunoassay (RIA).

**Isolated cells**

Testicular cell suspensions were prepared using the method of Stalvey & Payne (1983) as previously described (Murphy & O'Shaughnessy, 1991) but without further purification of the Leydig cells on Percoll gradients. The incubation time with collagenase which was required to disrupt the testes varied between groups, with control testes requiring 10 min, cryptorchid testes 15 min and testes from Tfm mice 25 min. Isolated cells were incubated for 3 h in M199 at 32 °C under 5% CO2 in air. Cells were incubated under non-stimulatory conditions (basal) or in the presence of hCG (200 mIU/ml) or dibutyryl cyclic AMP (dbcAMP) (2 mmol/l). Cell numbers were determined using a haemocytometer and the proportion of Leydig cells in each group measured by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (Payne, Downing & Wong, 1980). At the end of the incubations, cells plus medium were placed in a heating block at 100 °C for 5 min and then centrifuged at 1750g for 20 min. Supernatants were stored at −20 °C.

**Hormone assay**

Testosterone, progesterone and androstenedione were measured by individual RIAs (Sheffield & O'Shaughnessy, 1989; O'Shaughnessy, Pearce & Mannan, 1990). Intratesticular and serum testosterone concentrations were measured by RIA following extraction as previously described (O'Shaughnessy, Abbott, Leigh & Cattanach, 1991). Serum levels of bioactive luteinizing hormone (LH) were measured using a modified mouse in-vitro Leydig cell bioassay (Abbott, Hodges & George, 1988; O'Shaughnessy et al. 1991).
Pregnenolone metabolism

Testicular cells isolated from control, cryptorchid and Tfm mice were incubated, as above, for 3 h in the presence of 1 μCi [3H]pregnenolone. The steroid metabolites formed were extracted and separated as previously described (Mannan & O'Shaughnessy, 1988; Sheffield & O'Shaughnessy, 1988).

Steroidogenic enzyme activity

Whole testes were decapsulated and sonicated on ice (three or four bursts of 15 s with intervals of equal length) in phosphate buffer (pH 7·4; 50 mmol/l). Steroidogenic enzyme activity was measured by incubation of testicular homogenate with tritiated substrate and separation of products by TLC as previously described (O'Shaughnessy & Payne, 1982; O'Shaughnessy & Murphy, 1991). The substrate concentrations used for each enzyme were maximal as assessed using testes from normal mice.

3β-Hydroxysteroid dehydrogenase-isomerase

Activity of 3β-HSD was determined by measuring conversion of [3H]pregnenolone to [3H]progesterone. Incubations were for 5 min at 37°C in glass tubes containing [3H]pregnenolone (1 μCi, 2 μmol/l) dissolved in 0·03 ml dimethyl sulphoxide (DMSO) and 0·87 ml phosphate buffer containing NAD (1 mmol/l). The reaction was initiated by the addition of 0·1 ml homogenate and stopped by the addition of 0·1 ml NaOH (1 mol/l). [3H]Progesterone (50 μg, 1500 d.p.m.) was added to monitor recovery, and steroids were extracted with toluene. Pregnenolone and progesterone were separated by TLC in chloroform/ether (7/1, v/v) using plastic-backed silica gel plates (Whatman, Maidstone, Kent, U.K.). Radioactivity associated with each steroid was measured in a scintillation counter. Only progesterone is formed from pregnenolone under the conditions used to measure 3β-HSD activity in testes from normal and Tfm animals.

17α-Hydroxylase

Activity of 17α-hydroxylase was determined by measuring conversion of [3H]progesterone to [3H]17α-hydroxyprogesterone, [3H]androstenedione and [3H]testosterone during a 10-min incubation at 37°C. [3H]Progesterone (1 μCi, 1 μmol/l) was dissolved in DMSO in 0·87 ml phosphate buffer containing NADPH (1 mmol/l). The reaction was carried out as above and steroids were separated by TLC in chloroform/ether (7/1, v/v).

17-Ketosteroid reductase

Activity of 17-ketosteroid reductase was determined by measuring conversion of [3H]androstenedione (1 μCi, 10 μmol/l) to [3H]testosterone during a 10-min incubation in sodium acetate buffer (pH 5·0) containing NADPH (2 mmol/l). The extraction and separation procedures were as above.

5α-Reductase

Activity of 5α-reductase was determined by measuring conversion of [3H]testosterone (1 μCi, 2 μmol/l) to [3H]dihydrotestosterone and [3H]5α-androstane-3α,17β-diol. The incubation was for 20 min in phosphate buffer containing NADPH (0·5 mmol/l). Extraction and separation were as above.

Statistics

Results were analysed using t-tests or analysis of variance with the Neuman–Keul test. Metabolites formed from [3H]pregnenolone have been expressed as a percentage of the total [3H]pregnenolone metabolized. This removes differences in the total percentage of substrate metabolized due to differences in activity between samples.

RESULTS

Testosterone and LH levels

Serum and intratesticular concentrations of testosterone were significantly reduced in Tfm animals (Table 1) while serum bioactive LH levels were significantly increased (Table 1).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Serum LH (mIU/ml)</th>
<th>Serum (pmol/ml)</th>
<th>Intratesticular (pmol/testis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfm</td>
<td>24·4±5·0 (4)</td>
<td>13·7±4·8 (10)</td>
<td>4·4±0·3 (18)</td>
</tr>
<tr>
<td>Control</td>
<td>9·4±1·8 (6)</td>
<td>46·9±8·5 (11)</td>
<td>100±31·3 (12)</td>
</tr>
</tbody>
</table>

There was a significant (P<0·05) difference between Tfm and control animals in all parameters measured (t-test).

Testosterone production by whole testes

Testosterone produced by Tfm testes in vitro was markedly lower than control under basal conditions and in the presence of hCG (Fig. 1). Addition of hCG increased testosterone production by 30-fold in normal testes compared with only 1·7-fold in testes from Tfm mice.

Pregnenolone metabolism

To examine the effect of the Tfm mutation on the pattern of steroid metabolism, isolated testicular
Steroid production by isolated Leydig cells

Steroid production by Leydig cells isolated from testes of control, cryptorchid and Tfm mice is shown in Fig. 3. Androgen (androstenedione plus testosterone) production by Leydig cells from testes of Tfm mice was markedly lower than cells from control or cryptorchid testes under basal conditions and after trophic stimulation with hCG or dbcAMP (Fig. 3b). Progesterone production, however, was high in cells from Tfm testes and undetectable or only barely detectable in cells from control or cryptorchid testes (Fig. 3a).

Total steroid production (progesterone plus androstenedione plus testosterone) by Leydig cells from


testes of Tfm mice was significantly lower than cells from normal or cryptorchid testes during trophic stimulation with hCG or dbcAMP, although there was no difference under basal conditions (Fig. 3c). The pattern of steroid production was similar in Leydig cells from control and cryptorchid testes although androgen production and, therefore, total steroid production was significantly reduced in cells from the cryptorchid testes.

Steroidogenic enzyme activity

Activity of 3β-HSD did not differ significantly between testes from control, cryptorchid and Tfm mice (Table 2). In contrast, activity of 17α-hydroxylase was reduced in testes from Tfm mice to only 2-4% of control levels. Activity in cryptorchid testes was also significantly reduced but only by about 50% compared with control. Cryptorchidism also significantly reduced 17-ketosteroid reductase activity to about 15% of control levels (Table 2). In testes from Tfm mice, activity of this enzyme was reduced further to barely detectable levels (0-1% of control). Activity of 5α-reductase was low in all three groups and no significant difference was apparent.

DISCUSSION

The total insensitivity of the Tfm mouse to receptor-mediated androgen action (Ohno, 1971; Bardin et al. 1973) makes it an ideal model with which to study the function of androgens in normal development. Leydig cells contain androgen receptors (Verhoeven, 1980; Buzek & Sanborn, 1988) and a role for the hormone in normal Leydig cell development is likely. It has been shown previously that serum LH levels are high in the Tfm mouse but that this is associated with reduced serum testosterone (Goldstein & Wilson, 1972; Amador, Parkening, Beamer et al. 1986). Since circulating LH is biologically active in the Tfm mouse this suggested that there is a defect in testicular steroidogenesis. Results from this study show that lack of androgen production by the Tfm testis is due primarily to loss of 17α-hydroxylase activity although other enzymes, particularly 17-ketosteroid reductase, also appear to be affected by the mutation.

Testes in the Tfm mouse are intraabdominal and this condition itself inhibits 17α-hydroxylase activity by about 50%. It is clear, however, that a different mechanism must be responsible for the further loss of this enzyme in the Tfm mouse. Synthesis of the 17α-hydroxylase enzyme is regulated by receptor-mediated androgen action (Hales et al. 1987). While this regulation is negative in the normal adult it is possible that androgen receptor activity is required for synthesis of the 17α-hydroxylase enzyme during development. Alternatively, Sertoli cells are also known to have androgen receptors and it is already established that follicle-stimulating hormone can indirectly induce Leydig cell activity by its action on Sertoli cells (Kerr & Sharpe, 1985; Teerds, Closset, Rommerts et al. 1989). It is possible, therefore, that androgen action on the Sertoli cell may be required for normal Leydig cell activity.

It is possible, alternatively, that loss of 17α-hydroxylase activity in the Tfm mouse is only indirectly related to a lack of functional androgen receptors. Testosterone can act to decrease 17α-hydroxylase activity by a receptor-independent mechanism, causing oxygen-dependent degradation (Perkins, Hall & Payne, 1988). This effect, however, is only seen when testosterone is in high concentrations and it is unlikely to be responsible for the decreased 17α-hydroxylase in the Tfm mouse since intratesticular testosterone concentrations are less than normal. Synthesis of 17α-hydroxylase is dependent on LH (Purvis, Canick, Latif et al. 1973; Malaska & Payne, 1984) and it might normally be expected that the combination of abnormally high serum LH and low intratesticular testosterone would lead to increased levels of 17α-hydroxylase.

The 17α-hydroxylase gene is located on chromosome 10 in the human (Matteson, Picado-Leonard, Chung et al. 1986) and is unlikely, therefore, to be X-linked in the mouse. Loss of 17α-hydroxylase cannot, therefore, be explained by a mutation in the gene.

TABLE 2. Steroidogenic enzyme activity in homogenates of testes from normal, cryptorchid and Tfm mice. Values are means ± s.e.m. for the number of animals in parentheses

<table>
<thead>
<tr>
<th>Enzyme activity (pmol/min per testis)</th>
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<tbody>
<tr>
<td>3β-HSD</td>
</tr>
<tr>
<td>Tfm</td>
</tr>
<tr>
<td>Cryptorchid</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

Within each enzyme, groups with different letter subscripts are significantly (P < 0.05) different (Neuman–Keul test). 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17-KSR, 17-ketosteroid reductase.
It is possible, however, that there is a secondary mutation in the X chromosome of the Tfm mouse which affects expression of the 17α-hydroxylase gene. This may, for example, be a common transcription factor for both the androgen receptor and 17α-hydroxylase genes.

In addition to loss of 17α-hydroxylase, the testes from Tfm mice are grossly deficient in 17-ketosteroid reductase. A large part of this loss in activity can be explained by the cryptorchid state of the animal but it is clear that there is a further loss associated with the Tfm mutation. Lack of 17-ketosteroid reductase activity in the Tfm mouse has been reported previously (Blackburn et al. 1973) although the effect of cryptorchidism was not investigated. Studies on the Tfm rat have shown that this animal is also severely depleted in 17-ketosteroid reductase (Schneider & Bardin, 1970; Goldman & Klingele, 1974; Purvis, Clausen & Hansson, 1978). These results suggest that this enzyme may also be dependent on androgen receptor activity for normal development. Of the enzyme required for androgen synthesis in the testis, 17-ketosteroid reductase is the only one which shows significant activity in the seminiferous tubules (O'Shaughnessy & Murphy, 1991). Loss of 17-ketosteroid reductase activity in the testes of Tfm animals may, therefore, be due to lack of androgen action on Sertoli cells.

The loss of 17α-hydroxylase and 17-ketosteroid reductase in the Tfm mouse explains previous results from Goldstein & Wilson (1972) who showed that exogenous pregnenolone metabolism to testosterone is reduced in testes from Tfm mice. These results also explain the lack of effect of hCG on serum testosterone levels in Tfm mice reported by Amador et al. (1986). It is clear from results reported here that Leydig cell activity in the Tfm mouse can respond to hCG but that the major steroid produced is progesterone rather than testosterone.

Activity of 3β-HSD was normal in the Tfm testis and, since Leydig cell number is close to normal in these animals (Blackburn et al. 1973), this demonstrates that this enzyme is largely independent of androgen action. Activity of 5α-reductase shows a highly age-dependent pattern of activity in the normal rat and mouse with a peak around puberty in both species (Goldman & Klingele, 1974; Chase & Payne, 1983; Sheffield & O'Shaughnessy, 1988). In the Tfm rat activity remains high in the adult animal (Goldman & Klingele, 1974) although, from results described here, this is clearly different from the Tfm mouse. Activity in testes from Tfm mice was very low and not significantly different from normal and it is not clear why such a difference should arise between the two species.

We have reported previously that cryptorchidism, induced in the adult mouse, has an inhibitory effect on Leydig cell function (Murphy & O'Shaughnessy, 1991). Results reported here show that a similar effect is seen in adult animals if testicular descent is prevented at puberty. Cryptorchidism in these animals was associated with loss of steroidogenic enzyme activity and, in particular, 17-ketosteroid reductase. The mechanism underlying this loss of activity in the cryptorchid testis is unknown but may be related to the elevated temperature of the cryptorchid testis or to the loss of germ cells associated with this condition. The particularly high sensitivity of 17-ketosteroid reductase to cryptorchidism may be related to the delayed development of this enzyme in the normal testis (Payne, Kelch, Murono & Kerlan, 1977). In the rat, activity of most steroidogenic enzymes increases towards adult levels between 20 and 30 days (Goldman & Klingele, 1974; Payne et al. 1977), the time of normal testicular descent. Activity of 17-ketosteroid reductase, in contrast, does not rise until after descent is complete. It is possible, therefore, that a scrotal temperature is required for normal development of this enzyme.

We may conclude from the results reported here that the Tfm mutation is associated with severe loss of 17α-hydroxylase and 17-ketosteroid reductase activities. Pregnenolone metabolism to testosterone is normal in newborn Tfm mice (Goldstein & Wilson, 1972) which suggests that the defects in the steroidogenic pathway arise with development of the 'adult' generation of Leydig cells. Interestingly, it has been reported (Hardy, Kelce, Klinefelter & Ewing, 1990) that putative Leydig cell precursors in culture will only develop the capacity for androgen biosynthesis following androgen stimulation. Together, these results indicate a role for androgens in regulating normal development of Leydig cell function.

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REFERENCES


