J. Steroid Biochem. Molec. Biol. Vol. 39, No. 6, pp. 921–928, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

STEROIDOGENIC ENZYME ACTIVITY IN THE HYPOGONADAL (*hpg*) MOUSE TESTIS AND EFFECT OF TREATMENT WITH LUTEINIZING HORMONE

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(Received 12 March 1991)

Summary—The hypogonadal (*hpg*) mouse, which lacks circulating gonadotrophins during development, has been used (a) to determine whether initial expression of steroidogenic enzyme activity is dependent upon gonadotrophins and (b) to examine the responsiveness of these enzymes to luteinizing hormone (LH) stimulation. Activities of 17α-hydroxylase, 17-ketosteroid reductase and 5α-reductase were very low but detectable in the *hpg* testis while cholesterol side-chain cleavage (CSCC) activity was undetectable. In contrast, 3β-hydroxysteroid dehydrogenase (3βHSD) activity was high (11% of normal testis). Treatment with LH increased CSCC and 17α-hydroxylase activity more than 11-fold within 24 h. 5α-Reductase activity was increased 3-fold after 3 days treatment while 17-ketosteroid reductase and 3βHSD activities did not respond until after 10 days of treatment. The overall increases in 5α-reductase (4-fold) and 3βHSD (6-fold) activities were low while changes in 17-ketosteroid reductase (20-fold) and, particularly, CSCC (>130-fold) and 17α-hydroxylase (153-fold) were more marked. Results show (1) that expression of 3βHSD activity may be independent of gonadotrophins, (2) that activity of 17α-hydroxylase, 17-ketosteroid reductase and 5α-reductase is expressed, though at low levels, in the absence of gonadotrophins and (3) that prior exposure to gonadotrophins is not required for a rapid response to LH stimulation, particularly with respect to the cytochrome *P*-450 enzymes.

INTRODUCTION

Testosterone biosynthesis in the testis is dependent upon the activity of several steroidogenic enzymes located in the mitochondria and smooth endoplasmic reticulum (ser). Luteinizing hormone (LH) acts on the Leydig cell to cause acute stimulation of steroidogenesis but it is clear that this hormone is also involved in the longer-term control of steroidogenic enzyme activity. Early experiments demonstrated that hypophysectomy of adult rats causes a decrease in activity of the steroidogenic enzymes which is reversible by treatment with hCG [1, 2]. More recently it has been shown that withdrawal of LH from adult rats, using steroid implants, leads to similar reversible losses of enzyme activity [3, 4]. It is clear, therefore, that once enzyme activity has been established in the adult animal gonadotrophins are required to maintain activity. It is less clear, however, to what extent initial development of activity of the different steroidogenic enzymes is dependent upon the action of the gonadotrophins and to what extent activity is constitutively expressed within the Leydig cells.

The hypogonadal (*hpg*) mouse carries a deletion in the gene encoding for gonadotrophin-releasing hormone (GnRH) and its associated peptide [5]. As a consequence these animals lack GnRH, circulating levels of follicle-stimulating hormone (FSH) and LH are undetectable and the gonads develop in an environment lacking gonadotrophin stimulation [6]. Treatment of these animals for up to 2 weeks with GnRH, LH or testosterone stimulates testis growth while GnRH and LH stimulate androgen production [7–10]. In this study the *hpg* mouse has been used to examine steroidogenic enzyme activity in testes which have been deprived of gonadotrophin stimulation during development. In addition, the effects of LH treatment have been studied to determine the responsiveness of these enzymes to the onset of gonadotrophin stimulation.

EXPERIMENTAL

Materials

[4,7-^{3}H]pregnenolone, [1,2,6,7-^{3}H]progesterone, [1,2,6,7-^{3}H]androstenedione, [1,2,6,7-^{3}H]testosterone and ^{14}C-labelled steroids to

measure recovery were purchased from Amersham International (Amersham, Bucks., U.K.) and were purified by thin-layer chromatography (TLC) before use. [26,27-^{3}H]25-hydroxycholesterol was purchased from New England Nuclear (DuPont (U.K.) Ltd, Stevenage, Herts., U.K.). 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 and treatments

Normal and *hpg* mice were bred and reared at the Royal Veterinary College as previously described [10]. Adult *hpg* animals (90–150 days) were injected (s.c.) twice daily with 2 μg ovine LH (NIADDK-oLH-25; potency 2.3 × NIH-LH-S1) in saline or with saline alone. The FSH contamination of the LH used was less than 0.5% by weight. This dose of FSH has no effect on testicular growth or Leydig cell ultrastructure in *hpg* mice [9]. Animals were killed and used for experiments 12 h after the final injection.

Preparation of testicular homogenate

Animals were killed by decapitation and the testes rapidly removed into ice-cold buffer. For measurement of cholesterol side-chain cleavage (CSCC) activity testes were disrupted by homogenization in phosphate buffered saline (PBS) containing 0.1% bovine serum albumen (BSA) using a motor-driven Teflon-glass homogenizer. For measurement of other enzymes testes were disrupted by sonication in phosphate buffer (50 mM, pH 7.4). In most experiments tissue from each animal was treated independently but to measure CSCC activity in *hpg* animals it was necessary to pool testes from several animals (see below).

Measurement of steroidogenic enzyme activity

Cholesterol side-chain cleavage. Cholesterol side-chain cleavage activity was determined by measuring the conversion of side-chain labelled [26,27-^{3}H]25-hydroxycholesterol to tritiated 4-hydroxyl-4-methyl-pentanoic acid as previously described [11] and using an additional two-phase scintillation counting step [12]. Homogenates were prepared of single testes from normal animals or pooled testes from *hpg* animals. The number of testes in each pool depended on the LH treatment; in the untreated group 16–18 testes were used in a pool, after 1 days treatment between 8 and 16 testes were used, after 3 days treatment between 4 and 6 testes were used and after 10 or 20 days treatment homogenates were prepared from the paired testes of single animals. To measure CSCC activity homogenates were incubated in a total volume of 0.5 ml PBS containing 15 μl dimethyl sulphoxide, 5 mM isocitrate, 0.5 mM $NADP^+$, 0.1% BSA and 1 μCi [^{3}H]25-hydroxycholesterol (2 μM). Incubations were for 1 h at 37°C; in normal testes the reaction was shown to be linear during this time. At the end of the incubation 0.1 ml NaOH (1 N) was added followed by 0.5 ml phosphate buffer adjusted to pH 13. To remove non-metabolized substrate the mixture was extracted twice with chloroform and vortexed with alumina as described previously [11] and an aliquot was counted in a liquid scintillation counter using a toluene-based scintillant. The aqueous phase was then acidified to pH 1 using HCl and the vial recounted. The difference between the two counts represents [^{3}H]4-hydroxyl-4-methyl-pentanoic acid [12].

Activities of the other steroidogenic enzymes were measured by incubation of testicular homogenate with tritiated substrate and separation of products by TLC as previously described [13]. The substrate concentrations used for each enzyme were maximal as assessed using testes from normal mice.

3β-hydroxysteroid dehydrogenase-isomerase. Activity of 3βHSD was determined by measuring conversion of [^{3}H]pregnenolone to [^{3}H]progesterone. Incubations were for 10 min at 37°C in glass tubes containing [^{3}H]pregnenolone (1 μCi, 2 μM) dissolved in 0.03 ml dimethyl sulphoxide (DMSO) and 0.87 ml phosphate buffer containing NAD (1 mM). The reaction was initiated by the addition 0.1 ml homogenate and stopped by the addition of 0.1 ml 1 N NaOH. [^{14}C]Pregnenolone (50 μg, 1500 dpm) and [^{14}C]progesterone (50 μg, 1500 dpm) were added to monitor recovery and steroids were extracted with toluene. Pregnenolone and progesterone were separated by TLC in chloroform/ether (7/1) using plastic-backed silica gel plates (Whatman, Maidstone, Kent, U.K.). Radioactivity associated with each steroid was measured in a scintillation counter.

17α-hydroxylase. Activity of 17α-hydroxylase was determined by measuring conversion of [^{3}H]progesterone to [^{3}H]17α-hydroxyprogesterone, [^{3}H]androstenedione and [^{3}H]testosterone

during a 20 min incubation at 37°C. [^{3}H]Progesterone (1 μCi, 1 μM) was dissolved in DMSO in 0.87 ml phosphate buffer containing NADPH (1 mM). The reaction was as above and steroids were separated by TLC in chloroform/ether (7/1) [14].

17-Ketosteroid reductase. Activity of 17-ketosteroid reductase was determined by measuring conversion of [^{3}H]androstenedione (1 μCi, 10 μM) to [^{3}H]testosterone during a 25 min incubation in sodium acetate buffer (pH 5.0) containing NADPH (2 μM). The extraction and separation procedures were as above.

5α-Reductase. Activity of 5α-reductase was determined by measuring conversion of [^{3}H]testosterone (1 μCi, 2 μM) to [^{3}H]dihydrotestosterone and [^{3}H]5α-androstane-3α,17β-diol. The incubation was for 60 min in phosphate buffer containing NADPH (1 mM) and a generating system (1 mM D-glucose-6-phosphate plus 0.4 S.I./ml D-glucose-6-phosphate dehydrogenase). Extraction and separation were as above.

Statistics

Results were analysed by analysis of variance and Duncan's multiple-range test.

RESULTS

Testis weight

Twice daily injections of LH caused an increase in *hpg* testis weight which was first apparent after day 3 (Fig. 1). Under the treatment regime used testis weight reached a maximum at 10 days and did not change between 10 and 20 days.

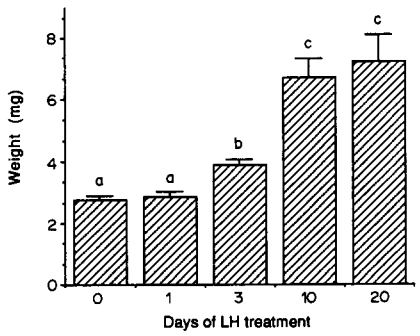

Fig. 1. Effects of LH treatment on testicular weight in the *hpg* mouse. The mean ± SEM is shown and the numbers of animals in each group were 18, 19, 19, 9 and 6 after 0, 1, 3, 10 and 20 days of LH treatment. Groups with different letter superscripts were significantly ($P < 0.05$) different.

Table 1. Steroidogenic enzyme activity in the normal and hypogonadal mouse

Enzyme	Enzyme activity (pmol/min/testis) Hypogonadal mouse	Normal mouse
CSCC	<0.008*	7.5 ± 0.6
3βHSD	237 ± 58	2088 ± 70
17α-Hydroxylase	0.45 ± 0.31	1040 ± 57
17-Ketosteroid reductase	2.01 ± 0.79	2040 ± 100
5α-Reductase	0.14 ± 0.03	Adult 0.98 ± 0.21 30-day old 9.2 ± 2.4

Mean ± SEM of three animals is shown for each group except CSCC activity in the *hpg* mouse. To measure this enzyme testes were pooled (up to 18 testes per pool) and activity in 3 separate pools determined. The animals used to study each enzyme were adult apart from the 5α-reductase enzyme in which both normal adult and 30-day old animals were used.

*Activity was undetectable and the limit of detection has been arbitrarily set at 20 times the background count in the scintillation counter used to measure activity.

Enzyme activity in hpg and normal mice

In the *hpg* testis the five steroidogenic enzymes measured showed markedly different activities (Table 1). Cholesterol side-chain cleavage activity was undetectable by the method used and activities of both 17α-hydroxylase and 17-ketosteroid reductase were extremely low. In contrast, 3βHSD activity was relatively high in the *hpg* mice. The difference between these enzymes may be seen in the ratio of 3βHSD activity to 17α-hydroxylase activity in normal mice (2/1) compared to *hpg* mice (525/1). Activity of 5α-reductase was also very low in *hpg* mice but activity of this enzyme is also low in adult normal mice. The peak activity of 5α-reductase in the mouse, as in the rat, is around puberty (Table 1).

Effect of LH on steroidogenic enzyme activity

(i) Cholesterol side-chain cleavage. Activity of CSCC was increased from undetectable levels (<0.008 pmol/min/testis) in control *hpg* mice to 0.103 pmol/min/testis by treatment with LH for 24 h (an increase of at least 12-fold) (Fig. 2). Activity continued to increase (by 10-fold) up to 10 days of LH treatment but declined between 10 and 20 days to 42% of the peak value.

(ii) 3β-Hydroxysteroid dehydrogenase/isomerase. Treatment of *hpg* mice with LH for 3 days had no effect on 3βHSD activity (Fig. 3A). By 10 days, however, there was a significant increase in enzyme activity which continued to rise until 20 days at which time activity in the *hpg* testis was similar to that in the testis of the normal animal (Table 1).

(iii) 17α-Hydroxylase. In contrast to 3βHSD, treatment of *hpg* mice with LH for 1 day caused an 11-fold increase in 17α-hydroxylase activity

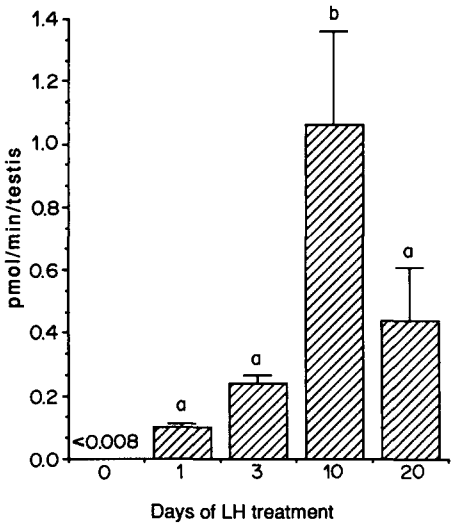

Fig. 2. Effect of treatment with LH for up to 20 days on CSCC activity in the *hpg* mouse. Animals, up to 9 per group, were injected twice daily with LH. Activity of CSCC was measured in homogenates of testes from single animals (after 10 or 20 days treatment) or in homogenates of pooled testes from several animals (see Experimental). Mean ± SEM is shown of results from 3 individual animals or 3 separate pools of testes in each group. Groups with different letter superscripts were significantly ($P < 0.05$) different.

(Fig. 3B). From 1 to 10 days there was a further 13-fold increase in activity but between 10 and 20 days of treatment there was a marked decline in 17α-hydroxylase activity to the levels seen after 1 and 3 days of treatment.

(iv) 17-Ketosteroid reductase. Treatment of *hpg* mice with LH had no effect on 17-ketosteroid reductase activity up to 3 days (Fig. 3C). Between 3 and 10 days there was an 18-fold increase in activity although this activity declined again by 65% between days 10 and 20 of treatment.

(v) 5α-Reductase. Activity of 5α-reductase in the *hpg* testis was unaffected by treatment with LH for 1 day but showed a 3-fold increase between 1 and 3 days (Fig. 3D). By 10 days the activity was similar to that seen in a normal adult animal (see Table 1). Between 10 and 20 days, however, activity declined again to the levels seen in control *hpg* animals.

DISCUSSION

In the Leydig cell, production of testosterone from pregnenolone depends upon the action of CSCC within the mitochondria and a further 3 enzymes associated with the ser; 3βHSD, 17α-hydroxylase and 17-ketosteroid reductase. Results described here show that in the *hpg* mouse testis, which lacks endogenous gonadotrophin stimulation during development, activities of all three enzymes associated with the ser are present indicating that the gonadotrophins are not required for at least a basal expression of their activity. Both 17α-hydroxylase and 17-ketosteroid reductase activities were extremely low, however, demonstrating that these enzymes are highly dependent upon the action of one or both of the gonadotrophins. In contrast, 3βHSD activity was relatively high in the control *hpg* mouse testes. The number of Leydig cells in an *hpg* testis is unknown but is likely to be lower than in a normal testis since Leydig cell number goes through a marked increase around the time of puberty, at least in the rat [15]. Thus activity of 3βHSD per Leydig cell may be close to normal in the *hpg* mouse. This suggests that expression of 3βHSD activity contains a component which is largely independent of the action of the gonadotrophins. In the rat, withdrawal of LH from the adult animal using steroid implants causes a significant decrease in activity of steroidogenic enzymes with the exception of 3βHSD activity which remains unchanged [3, 16]. Similarly, testicular 3βHSD appears to be relatively resistant to hypophysectomy [1, 3] although some reports show a marked decline in activity [17, 18]. These results and studies reported here suggest that activity of 3βHSD in the testis may either be largely constitutively expressed or regulated by factors other than the pituitary hormones.

In contrast to other enzymes studied CSCC was undetectable in control *hpg* testes. The method used to measure CSCC activity is not ideal for use in the mouse since, unlike the rat, the affinity of the mouse CSCC enzyme for 25-hydroxycholesterol is not as high as for the endogenous substrate [19]. This method was used, however, since hydroxylated sterols have advantages over cholesterol in the measurement of CSCC activity [19, 20] and other straightforward methods of measuring CSCC in the rat are not applicable to the mouse [21]. The lack of detectable CSCC in the control *hpg* testes should not, therefore, be taken to indicate that activity is absent in these animals. These results clearly show, nevertheless, that in the absence of significant gonadotrophin stimulation during development expression of CSCC is extremely low. This is in contrast to Leydig cells from normal adult mice which continue to express CSCC activity in culture in the absence of hormonal stimulation [22]. It is possible, therefore, that constitutive expression of significant

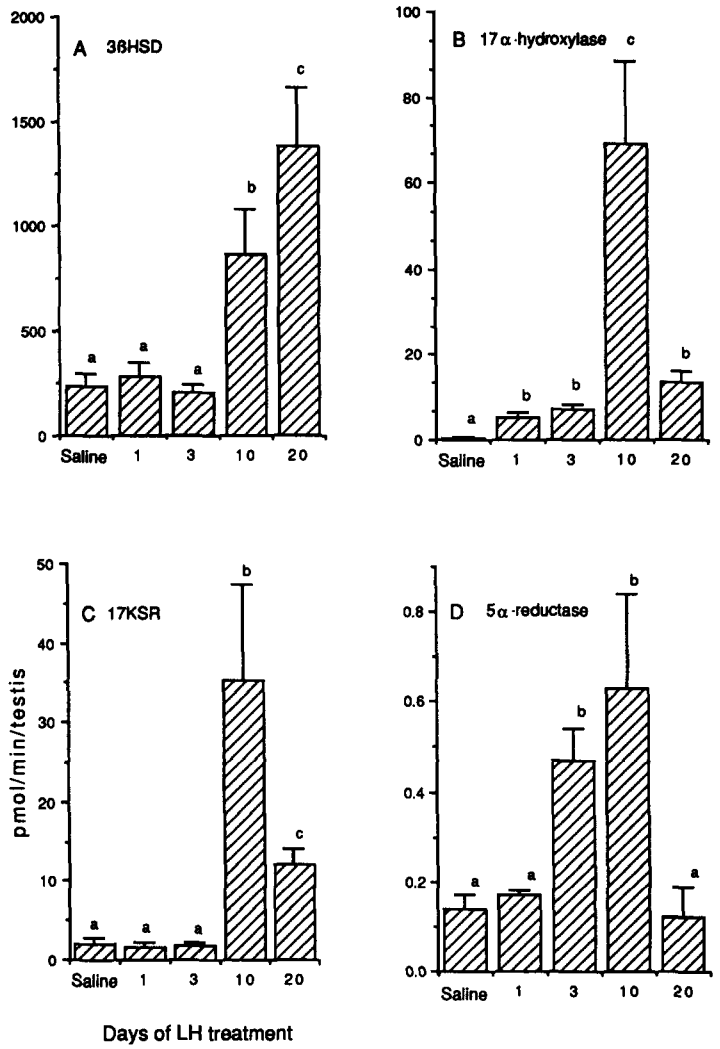

Fig. 3. Effect of LH treatment on steroidogenic enzyme activity in the *hpg* mouse. Animals (3 per group) were injected with LH for up to 20 days and activity of 3β HSD (A), 17α-hydroxylase (B), 17-ketosteroid reductase (17KSR) (C) and 5α-reductase (D) measured. Control animals were treated with saline for 10 days, there was no significant difference between control *hpg* animals and animals treated with saline (data not shown). The mean ± SEM is shown. Groups, within each enzyme studied, with different letter superscripts were significantly ($P < 0.05$) different.

CSCC activity in mouse Leydig cells is dependent upon prior exposure of the cells to gonadotrophin. Results described here also show that CSCC activity in the *hpg* testis is highly sensitive to the effects of LH with a marked increase (> 12-fold) in activity within 24 h of injection.

In early studies by Purvis *et al.* [2] it was demonstrated that hypophysectomy causes a rapid decline in 17α-hydroxylase activity in the rat testis and that hCG could restore activity. More recently it has been shown using mouse Leydig cell cultures that LH or cyclic AMP is required to induce and maintain 17α-hydroxylase activity [22, 23]. Results reported here show that there is a very low level of 17α-hydroxylase activity expressed in the absence of gonadotrophins and that the Leydig cells of the *hpg* testis are highly sensitive to the effects of LH since there was an 11-fold increase in activity after 1 day of treatment. These results show that the two cytochrome *P*-450 enzymes studied, CSCC and 17α-hydroxylase, are highly dependent upon LH for activity and are also highly sensitive to the effects of the hormone despite the lack of exposure to endogenous LH during development.

17-Ketosteroid reductase activity in the *hpg* testis was less sensitive to the effects of LH than CSCC or 17α-hydroxylase, a significant increase in activity only being detectable after 3 days. The magnitude of this response to LH was, however, considerably greater in the *hpg* testis

than in testes of the hypophysectomized rat [2]. The lack of sensitivity of 17-ketosteroid reductase to hCG in the hypophysectomized rat may indicate a species difference or that other pituitary hormones play a synergistic role, along with LH, in controlling 17-ketosteroid reductase activity.

In the rat 5α-reductase activity shows a highly age-dependent pattern of expression with a peak of activity around 30 days [24]. Chase and Payne [25] have also shown that in the mouse there is increased Leydig cell production of androstanediol around this time. Results reported here confirm that testicular 5α-reductase activity is nine times higher in the pubertal mouse than in the adult animal. Following EDS-induced destruction of Leydig cells in the adult rat the regenerating population of Leydig cells show a pattern of 5α-reductase activity similar to that seen in pubertal animals [26, 27]. This has led to the suggestion that during testicular development there is predetermined regulation of 5α-reductase. Results from the present study show that LH treatment of *hpg* animals will increase 5α-reductase activity but only to levels seen in the adult and not to those of the pubertal animal. LH has been shown to regulate 5α-reductase activity in immature rats [28–30] and the increased activity may be due to direct effects of LH or to an increase in Leydig cell number (see below). There is no evidence, however, from these studies of high constitutive expression of 5α-reductase during Leydig cell development.

After 10 days treatment of *hpg* mice with LH there is evidence of Leydig cell hyperplasia [9] and it may be expected that Leydig cell number will increase during this time. It is likely that the early effects of LH, seen after 1 and 3 days, are due, largely, to changes in enzyme activity per cell. Later changes in enzyme activity after 10 days may be partly due, in contrast, to changes in cell number. It is possible, therefore, that the 6-fold increase in 3βHSD activity after 20 days treatment is due to increased Leydig cell number. If the increased activity of this enzyme is assumed to be due entirely to changes in Leydig cell number this sets a possible limit for the increase in cell number of 6-fold. The significantly greater effects of LH on CSCC, 17α-hydroxylase and 17-ketosteroid reductase over 10 days are, therefore, much more likely to be due to changes in activity per Leydig cell rather than simply changes in cell number.

Between 10 and 20 days of LH treatment there was a decline in activity of the steroidogenic enzymes with the exception of 3βHSD. This effect may be due to any of several possible mechanisms including desensitization of the testis to LH, development of anti-LH antibodies or down regulation by high intra-testicular androgen concentrations. Treatment of rats with high doses of LH or hCG profoundly inhibits testicular steroidogenesis and decreases the activity of 17α-hydroxylase [13, 31, 32]. These effects tend to be rapid, however, and it is likely that they would be manifest before 10 days. In addition, it remains to be shown whether mouse testes are desensitized over the long term by treatment with LH or hCG. The decline in enzyme activity in the *hpg* testis between 10 and 20 days of LH treatment may, alternatively, be due to development of antibodies to the injected ovine LH. Neutralization of the injected LH would explain the decline in activity of most of the steroidogenic enzymes while the maintenance of 3βHSD activity may be due to the insensitivity of this enzyme to changes in circulating LH. A third factor which may cause an inhibition of enzyme activity is variation in intra-testicular androgen levels. Treatment of *hpg* animals with LH for 10 days causes a marked rise in intra-testicular androgen [9] and it is known that androgens show receptor-mediated, inhibitory effects on testicular steroidogenesis [33–35]. This effect is well described for 17α-hydroxylase [36] although it is unclear to what extent other enzymes are affected by changes in intra-testicular androgens.

Of the enzymes required for testosterone biosynthesis only 3βHSD activity reached apparently normal levels after the treatment regime used in this study. This failure to reach normal levels is likely to be due to one or more of several factors including hormone concentration, injection frequency and requirement for FSH. Variations in dose and injection frequency have been shown to have marked effects on the responsiveness of the *hpg* pituitary and gonads to GnRH [7] and it is likely that these factors will affect testicular responsiveness to the gonadotrophins. It is unlikely that FSH has direct effects on steroidogenic enzyme activity since, with the exception of 17KSR, all of the enzymes required to synthesize testosterone are found, almost exclusively, in the Leydig cells of the adult testis [37]. Nevertheless, there is convincing evidence that FSH will affect Leydig cell

activity through action on the Sertoli cell [38, 39] and FSH may be required for full expression of steroidogenic enzyme activity.

In summary, these results demonstrate that the steroidogenic enzymes show a basal constitutive level of activity in the mouse Leydig cell even without gonadotrophin stimulation during development. These results also show that the cytochrome *P*-450 enzymes CSCC and 17α-hydroxylase are highly sensitive to LH, again without prior exposure to the hormone, while 3βHSD activity appears to be expressed largely independently of gonadotrophin stimulation.

Acknowledgements—This study was supported by grants from the Medical Research Council and the Wellcome Trust. Supply of hormone by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the National Hormone and Pituitary Program (University of Maryland School of Medicine) is gratefully acknowledged.

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