Maintenance or stimulation of steroidogenic enzymes and testosterone production in rat Leydig cells by continuous and pulsatile infusions of luteinizing hormone during passive immunization against gonadotrophin-releasing hormone

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Summary. The importance of the pulsatility of luteinizing hormone (LH) secretion in maintaining key enzymes in the testosterone biosynthetic pathway in Leydig cells was studied using rats in which LH secretion was suppressed by passive immunization against gonadotrophin-releasing hormone (GnRH) and replaced by continuous or pulsatile i.v. infusions of exogenous LH, all delivering the same daily dose of the hormone (300 ng per 100 g NIDDK-ovine LH-24). Continuous infusions (12.5 ng per 100 g h⁻¹) were compared with infusions of 1 min pulses every 2 h (25 ng per 100 g) and every 4 h (50 ng per 100 g). After 5 days of treatment *in vivo* with sheep anti-GnRH serum (or normal sheep serum) and LH (or vehicle), Leydig cells were purified and assayed *in vitro* for maximum production of testosterone stimulated by human chorionic gonadotrophin (hCG) and supported by 25-hydroxycholesterol and for the activities of cholesterol side-chain cleavage, Δ^5 -3 β -hydroxysteroid dehydrogenase- Δ^5 -4-isomerase (3 β -HSD-isomerase) and 17 α -hydroxylase. Relative contents of cholesterol side-chain cleavage and 17 α -hydroxylase were also quantified by western and immunoblotting analysis. Activity of 3 β -HSD-isomerase was reduced by about 40% by anti-GnRH treatment and was increased by all LH regimens in anti-GnRH-treated animals, with no consistent pattern in the effects of the different LH regimens. Results for testosterone-producing capacity and the other two enzymes differed in several respects. Treatment with anti-GnRH serum markedly reduced basal, hCG-stimulated and 25-hydroxycholesterol-supported testosterone production (by 80–90%) and the activities of cholesterol side-chain cleavage (about 80%) and 17 α -hydroxylase (about 65%). Infusion of exogenous LH in any of the regimens tested prevented these changes or increased the activities to values greater than those in normal serum-treated controls. Differences in immunodetectable contents of the two enzymes generally paralleled those in enzyme activities. There was a consistent trend in the effects of LH replacement regimens on these parameters of steroidogenic activity: continuous infusions were more effective than pulses at 2 h intervals and these in turn were more effective than pulses at 4 h intervals, suggesting that the frequency of LH exposure is more important than the amplitude of individual exposures in maintaining Leydig cell steroidogenic function. Consistent differences among groups in hCG-stimulated relative to 25-hydroxycholesterol-supported testosterone production suggest that some constituents of Leydig cells prior to cholesterol side-chain cleavage enzyme are more sensitive to LH withdrawal and deviations from 'optimal' LH exposure than are the side-chain cleavage and subsequent enzymes in the testosterone biosynthetic pathway.

Keywords: Leydig cells; testosterone; steroidogenesis; P-450; luteinizing hormone; rat

Introduction

The steroidogenic capacity of testicular Leydig cells is normally maintained by exposure to pulsatile secretion of luteinizing hormone (LH). Previous studies in our laboratory have shown, however, that maintenance of the steroidogenic capacity of Leydig cells does not depend on the cells being exposed to the hormone intermittently, as they are normally by pulsatile LH secretion (Chase *et al.*, 1988; Gibson-Berry & Chase, 1990). In adult rats, for example, continuous intravenous infusions of LH were as effective as pulsatile infusions of the same daily dose (given as a series of 1 min pulses at intervals of 2 h) in preventing the reduction in steroidogenic capacity of Leydig cells following suppression of LH secretion by passive immunization against gonadotrophin-releasing hormone (GnRH) (Gibson-Berry & Chase, 1990). The fact that changes in production of testosterone supported by 25-hydroxycholesterol paralleled those in testosterone production stimulated by human chorionic gonadotrophin (hCG) or cyclic AMP suggested that the changes were associated with changes in the activities of enzymes catalysing conversion of cholesterol to testosterone. The present studies used the same model to study regulation of the activities and contents of three of these enzymes: (i) cholesterol side-chain cleavage, the mitochondrial cytochrome P-450 enzyme that catalyses conversion of cholesterol to pregnenolone (commonly designated as P-450_{SCC}); (ii) 3 β -hydroxysteroid dehydrogenase- Δ^5 - Δ^4 -isomerase (3 β -HSD-isomerase), a non-P-450 enzyme of the smooth endoplasmic reticulum that catalyses conversion of pregnenolone to progesterone; and (iii) 17 α -hydroxylase, a cytochrome P-450 localized in the smooth endoplasmic reticulum that catalyses conversion of progesterone to 17 α -hydroxyprogesterone (commonly designated as P-450_{17 α}). Biochemical, cloning and transfection studies have indicated that the single protein P-450_{17 α} catalyses the 17,20-lyase reaction, as well as the hydroxylation, to form androstenedione (reviewed by Hall, 1986 and Waterman & Simpson, 1989).

Most studies indicate that all these enzymes are dependent on LH for maintenance of normal activity in Leydig cells (e.g. Samuels & Helmreich, 1956; Menon *et al.*, 1967; Shikita & Hall, 1967; Purvis *et al.*, 1973a, b; Muroto & Payne, 1979; Shaw *et al.*, 1979; O'Shaughnessy & Payne, 1982; Ewing *et al.*, 1983), but there are several exceptions. Results by Ewing *et al.* have suggested that 3 β -HSD-isomerase is less dependent on LH than are the other enzymes: the ability of whole perfused rat testes to produce progesterone from pregnenolone was unaffected by 10 days of LH withdrawal by either hypophysectomy or subcutaneous implants of testosterone and oestradiol (Wing *et al.*, 1985) and was reduced by only 75% after 16 weeks of treatment with the steroid implants (Keeney *et al.*, 1988). Payne and colleagues have reported that the content of P-450_{SCC} protein as well as its rate of *de novo* synthesis and the content of its mRNA are maintained constitutively at high values in mouse Leydig cells cultured in the absence of LH or cAMP (Anakwe & Payne, 1987; Hales & Payne, 1989). Although there are these conflicting results on the degree to which the enzymes are strictly dependent on LH, there seems to be no dispute among the reports cited that the gonadotrophin can increase the activities of all three enzymes.

The present studies examined the LH dependence of the three enzymes in the *in vivo* model in which LH secretion is suppressed by passive immunization against GnRH, and determined whether the mode of LH administration (continuous versus pulsatile) in this model influences the response of any of the enzymes to the hormone. Experiments also compared testosterone production stimulated by gonadotrophin and supported by exogenous substrate *in vitro* as an indication of differential effects of *in vivo* treatments on constituents prior to these enzymes compared with effects on the enzymes themselves.

Materials and Methods

Animals, anti-GnRH immunizations and LH infusions

Young adult Sprague-Dawley rats (3–4 months old) were housed under a photoperiod of 12 h of light and 12 h of darkness and given Purina rat chow and water *ad libitum*. All experimental treatments were for 5 days. Two days

before the beginning of the experiment, intra-atrial cannulae of Silastic tubing were inserted through the right internal jugular vein, brought out through the back of the neck and connected to automatic infusion pumps with custom-made Teflon-brass swivels, as described by Gibson-Berry & Chase (1990). Surgical anaesthesia was achieved with i.m. injections of ketamine-HCl (50 mg kg⁻¹) and xylazine (10 mg kg⁻¹) and rats were given 60 000 iu penicillin-G (procain salt) i.m. to prevent infection. After surgery and throughout the experiment, drinking water was supplemented with aspirin (40 mg l⁻¹) to inhibit clotting of the cannulae.

Rats were treated with sheep anti-GnRH serum (Chase *et al.*, 1988) to suppress endogenous LH secretion (or with normal sheep serum) and infused with exogenous LH (or vehicle) for 5 days. The antiserum used in these studies was from a single bleeding of a single ram and was of high titre, binding about 60% of ¹²⁵I-labelled GnRH (100 pmol l⁻¹) at a final dilution of 1:100 000. (See Chase *et al.*, 1988, for descriptions of GnRH radioiodination, binding assay, etc.) Rats were given 2 ml of antiserum through intra-atrial cannulae on the first day of the experiment, and 1 ml every day thereafter. Pilot studies showed that this regimen effectively suppressed endogenous LH secretion, as serum testosterone was always reduced to castrate concentrations within several hours of the first antiserum infusion and was still at those concentrations immediately before any subsequent antiserum infusion. Control rats received the same regimen of infusions of normal sheep serum.

Starting 2 h after the first serum infusion, antiserum-treated rats were infused through the intra-atrial cannulae with vehicle (Krebs-Ringer containing 0.5 mg glucose ml⁻¹, 20 iu heparin ml⁻¹ and 1 mg bovine serum albumin (BSA) ml⁻¹, pH 7.2) or with NIDDK ovine LH-24 (biological potency = 2.3 × NIH LH S1) in vehicle at a total daily dose of 300 ng per 100 g, given as continuous infusions (at 12.5 ng per 100 g h⁻¹) or as discontinuous infusions of 1 min pulses at intervals of 2 h (25 ng per 100 g) or 4 h (50 ng per 100 g). Rats treated with normal sheep serum were infused with vehicle given as pulses every 2 h. All rats received the equivalent of 0.1 ml of vehicle h⁻¹. All infusions were delivered by means of electronic infusion pumps equipped with antisiphoning restraints to prevent syringe plungers from advancing unless the pumps were running. For pulsatile regimens, pumps were controlled by automatic timers customized to provide precise durations of 'on' events using appropriate circuits and relays. Fresh LH solutions were prepared every other day from frozen aliquots of a concentrated stock solution (20 mg LH ml⁻¹ Dulbecco's phosphate-buffered saline containing 10 mg BSA ml⁻¹). Rats were killed 5 days after the start of treatment, at the time of the next scheduled pulse of LH (or vehicle) or immediately after cessation of continuous infusions.

Purification of Leydig cells

Testes were dissociated by incubation with collagenase (1 mg ml⁻¹) in tissue culture medium 199 (TCM199) + 10 mmol Hepes l⁻¹ + 0.5% BSA, and Leydig cells were purified by Percoll gradient centrifugation, as described by Gibson-Berry & Chase (1990). Final purified cell suspensions contained 70–80% Leydig cells as identified by histochemical staining for 3β-hydroxysteroid dehydrogenase (after Payne *et al.*, 1980). Final cell suspensions were in a 1:1 mixture of Dulbecco's modified Eagle's medium:Ham's F12 + 10 mmol Hepes l⁻¹ + 0.1% BSA (DME:F12); this was used (rather than TCM199) so that the incubation medium in cholesterol side-chain cleavage assays would be devoid of exogenous cholesterol.

Testosterone production *in vitro*

Aliquots of purified Leydig cells were incubated for 1 h at 34°C in a shaking water bath (1 cycle s⁻¹) under an atmosphere of 95% O₂:5% CO₂. Incubations were in a total volume of 1.0 ml, containing no additions (basal), a maximally stimulatory concentration of hCG (50 miu ml⁻¹), or a saturating concentration of 25-hydroxycholesterol (10 nmol ml⁻¹), made up with 0.1 ml of cell suspension in DME:F12 and 0.9 ml of TCM199 + 10 mmol Hepes l⁻¹ + 0.1% BSA. Dimethylsulfoxide (7 μl ml⁻¹ = 100 μmol l⁻¹) was included to solubilize 25-hydroxycholesterol. This analogue was used because it can serve as substrate for testosterone production independently of the cyclic-AMP-dependent mechanisms that transport the endogenous substrate cholesterol to the mitochondria and increase its association with cholesterol side-chain cleavage enzyme (Toaff *et al.*, 1979, 1982; Georgiou & Payne, 1985; Quinn *et al.*, 1985). Incubations were terminated by chilling samples to 4°C, samples were centrifuged (1000 g for 20 min) and supernatants were decanted and assayed for testosterone by radioimmunoassay (Gibson-Berry & Chase, 1990).

Enzyme activity assays

For assays of 17α-hydroxylase and 3β-HSD-isomerase, Leydig cells were disrupted by ultrasonication and incubated for 5 min at 37°C with saturating concentrations of [1,2-³H]-progesterone (2 μmol l⁻¹) and NADPH (0.5 mmol l⁻¹) and with saturating concentrations of [1,2-³H]-pregnenolone (2 μmol l⁻¹) and NAD (0.5 mmol l⁻¹), respectively, essentially as described by O'Shaughnessy *et al.* (1981) and O'Shaughnessy & Payne (1982). Incubations were in a total volume of 1.0 ml, made up with 0.1 ml of disrupted cell suspension in DME:F12 and 0.9 ml of 0.05 mol potassium phosphate-buffered saline l⁻¹ (0.15 mol NaCl l⁻¹), pH 7.4, containing 0.05 ml dimethylsulfoxide (to solubilize substrates) and appropriate concentrations of substrate and cofactor. Incubations were terminated by

adding 0.1 ml of NaOH (1 mol l^{-1}) and chilling to 4°C . Blanks, in which enzyme activity was inhibited by addition of NaOH before addition of disrupted cells, were included with each assay. Appropriate carriers ($25 \mu\text{g}$) and ^{14}C -labelled recovery standards (600–800 d.p.m.) were added: 17α -hydroxyprogesterone, androstenedione and testosterone in the case of 17α -hydroxylase, and progesterone in the case of 3β -HSD-isomerase. Samples were extracted with 10 ml of toluene, extracts were evaporated and redissolved in methanol, and products and substrates were separated by thin-layer chromatography. The solvent systems were benzene:ethyl acetate (2:1, v:v) for the 17α -hydroxylase assay, and chloroform:diethyl ether (7:1, v:v) for the 3β -HSD-isomerase assay. In the former assay, the only product consistently above blank values was 17α -hydroxyprogesterone; all results are expressed, therefore, in terms of this product alone. The spot corresponding to this product was eluted with methanol and subjected to a second chromatography (after addition of additional carrier progesterone) to minimize contamination with reaction substrate. The only product in the 3β -HSD-isomerase assay was progesterone. Content of ^{3}H -products in thin-layer chromatograms was quantified by liquid scintillation spectrometry and corrected by recovery of ^{14}C -labelled recovery standards.

Cholesterol side-chain cleavage activity was assayed by incubating intact cells for 1 h at 34°C under 95% O_2 :5% CO_2 with a saturating concentration of $[26,27\text{-}^3\text{H}]\text{-25-hydroxycholesterol}$ ($10 \mu\text{mol l}^{-1}$) and determining ^{3}H -isocaproic acid, as described by Georgiou *et al.* (1987). Incubations were in a total volume of 1.0 ml of DME:F12, made up of 0.1 ml of cell suspension and 0.9 ml of medium containing substrate and $7 \mu\text{l}$ of dimethylsulfoxide ($100 \mu\text{mol l}^{-1}$) to solubilize the substrate. Incubations were terminated by adding 0.1 ml of NaOH (1 mol l^{-1}) and chilling to 4°C , and 0.1 ml of $[^1\text{C}]\text{-caproic acid}$ in H_2O was added to monitor recovery of product. Samples were centrifuged ($1000 g$ for 20 min), supernatants were decanted, pellets were washed with 1.0 ml of basified medium and recentrifuged, and these supernatants were combined with those from the first centrifugation. Supernatants were extracted with 10 ml of chloroform (to remove unreacted substrate) and aliquots (1.5 ml) of aqueous phases were mixed with aluminium oxide (0.8 g, to extract more unreacted substrate). Content of ^{3}H -isocaproic acid was determined by liquid scintillation spectrometry of aliquots (0.4 ml) of the final aqueous phases, and corrected by recovery of $[^1\text{C}]\text{-caproic acid}$. This method of determining cholesterol side-chain cleavage activity has been thoroughly validated for rat Leydig cells by confirming that results are the same when activity is measured as the formation of $[16,17\text{-}^3\text{H}]\text{-pregnenolone}$ and -progesterone from $[16,17\text{-}^3\text{H}]\text{-25-hydroxycholesterol}$ or the formation of pregnenolone and progesterone (measured by radioimmunoassay) from 25-hydroxycholesterol, in the presence of the 17α -hydroxylase inhibitor SU-10603 in both cases (Georgiou *et al.*, 1987).

Electrophoresis, western blotting and immunoblotting analysis of enzyme content

Aliquots of purified cells were washed twice with 0.01 mol sodium-phosphate-buffered saline (NaPBS) l^{-1} , using 15 min centrifugations at $500 g$ to pellet cells. Final cell pellets were lysed with 1% cholate and 0.1% sodium dodecyl sulfate (SDS) in NaPBS (6×10^6 cells ml^{-1}) and stored at -20°C for polyacrylamide gel electrophoresis (PAGE). Lysates were denatured by adding 0.5 volume of 6% SDS + 100 mmol dithiothreitol l^{-1} + 25% sucrose + 0.02% bromphenol blue in NaPBS and heating in boiling water for 15 min. Volumes of sample representing 60 000 and 100 000 Leydig cells were subjected to electrophoresis in 10% polyacrylamide gels for 17α -hydroxylase (P-450_{17 α}) and cholesterol side-chain cleavage enzyme (P-450_{SCC}), respectively. Proteins were transferred electrophoretically from gels to polyvinylidene fluoride membranes, using 25 mmol Tris-HCl litre^{-1} + 192 mmol glycine l^{-1} , pH 8.3, + 15% methanol (modified from Towbin *et al.*, 1979, and Burnette, 1981, as recommended by the manufacturer). Membranes were incubated for 1 h at room temperature in 20 mmol Tris-HCl litre^{-1} + 0.15 mol NaCl litre^{-1} , pH 7.4 (TNS), containing 5% BSA + 5% non-fat dry milk to block nonspecific binding. Membranes were washed in TNS and incubated overnight at 4°C with rabbit anti-bovine adrenocortical P-450_{SCC} or anti-porcine testicular P-450_{17 α} antiserum diluted in TNS containing 0.05% Tween-20. Use of these antisera to quantitate the enzymes in rat and mouse Leydig cells has been validated by Payne and colleagues (Anakwe & Payne, 1987; Georgiou *et al.*, 1987; Perkins & Payne, 1988; Hales & Payne, 1989). Membranes were washed with TNS containing 0.05% Tween-20, and bound antibodies were labelled by incubation for 3 h at 4°C with ^{125}I -labelled protein A (prepared by the chloramine-T reaction and Sephadex G-25 chromatography). Membranes were then washed, dried and exposed to Kodak X-Omat AR film with Cronex intensifying screens at -70°C . Autoradiograms were developed with Ilford Bromophen developer.

Apparatus, supplies and reagents

Intra-atrial cannulae were made of medical grade Silastic tubing (i.d. 0.025 inches; o.d. 0.047 inches; from Dow Corning Corp., Midland, MI, USA). Infusion pumps were model A99 from Razel Scientific Instruments, Inc. (Stamford, CT, USA). Electronic timers were model T1975 from Intramatic, Inc. (Spring Grove, IL, USA) with circuits and relays from Allied Electronics, Inc. (Elgin, IL, USA). Ketamine-HCl was Ketaset from Bristol Labs (Syracuse, NY, USA). Xylazine and penicillin-G were obtained from Sigma Chemical Co. (St Louis, MO, USA). The LH preparation used in these experiments was NIDDK ovine LH-24 (biological potency = $2.3 \times \text{NIH-LH-S1}$). Human chorionic gonadotrophin (hCG; 3000 iu mg^{-1}), fatty-acid-free BSA, Hepes, Percoll, neutral chromatographic aluminium oxide (type WN-9), chloramine-T, Dulbecco's modified Eagle's medium, and Ham's nutrient mixture F12 were obtained from Sigma. Tissue culture medium 199 was from Gibco (Grand Island, NY, USA). Collagenase (type A) and dithiothreitol were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Acrylamide

(4 × recrystallized), sodium cholate and sodium dodecyl sulfate were obtained from Serva Biochemicals (Westbury, NY, USA). *N,N'*-methylene-bisacrylamide was from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Glycine, protein A, tris(hydroxymethyl)-aminomethane (Tris), sucrose, bromphenol blue, and Tween-20 were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Sephadex G-25 was from Pharmacia Inc. (Piscataway, NJ, USA). Cronex intensifying screens, carrier-free Na^{125}I , $[7\text{-}^3\text{H}]\text{-pregnenolone}$ (25 Ci mmol^{-1}), $[1,2\text{-}^3\text{H}]\text{-progesterone}$ (55 Ci mmol^{-1}), $[26,27\text{-}^3\text{H}]\text{-25-hydroxycholesterol}$ (87 Ci mmol^{-1}), and $[4\text{-}^{14}\text{C}]\text{-progesterone}$, $17\alpha\text{-hydroxy-progesterone}$, -androstenedione , and -testosterone ($50\text{--}60\text{ mCi mmol}^{-1}$) were purchased from DuPont-New England Nuclear (Boston, MA, USA). Radiolabelled steroids were repurified by thin-layer chromatography before use. $[^{14}\text{C}]\text{-Caproic acid}$ was obtained from Research Products International Corp. (Mt Prospect, IL, USA). Nonradioactive steroids were obtained from Sigma, except for 25-hydroxycholesterol, which was from Steraloids, Inc. (Wilton, NH, USA). All organic solvents were high-performance liquid chromatography grade obtained from Baker Chemical Co. (Phillipsburg, NJ, USA), except for the following: diethyl ether was anaesthetic grade from Baker; dimethylsulfoxide was spectrophotometric grade from Aldrich and toluene was 'scintanalsed' from Fisher. Liquid scintillation cocktails were ScintiVerse E (Fisher) for aqueous samples and Liquifluor (DuPont-NEN) for spots from thin-layer chromatograms. Thin-layer chromatography of steroids was on ITLC-SA sheets from Gelman Scientific Co. (Ann Arbor, MI, USA). Polyvinylidene fluoride membranes were Immobilon P from Millipore Corp. (Bedford, MA, USA). Rabbit antisera against bovine adrenocortical P-450_{scc} and porcine testicular P-450_{17 α} were gifts from A. H. Payne (University of Michigan, Ann Arbor, MI, USA). Rabbit antiserum for testosterone radioimmunoassay was from G. M. Connell (Berkeley, CA, USA).

Statistical analyses

Analyses were carried out using analysis of variance and Newman-Keul's test after logarithmic transformation of data. Results are depicted as mean $\pm$ SEM and values for a given variable that are significantly different among groups ($P = 0.05$) are denoted by different lower-case letters. There were six or seven animals in each group.

Results

Treatment with anti-GnRH serum reduced basal and maximum hormone-stimulated and substrate-supported testosterone production by 80–90%; concurrent infusion of LH in any of the three regimens prevented these reductions in steroidogenic activity or increased the activity to values even greater than those in normal serum-treated controls (Fig. 1). Although not all the differences among groups of LH-treated rats were statistically significant, there was a consistent trend for the effectiveness of LH replacement regimens in maintaining steroidogenic activity to vary directly with frequency (inversely with amplitude) of LH administration (i.e. continuous infusions were more effective than pulses at 2 h intervals and these in turn were more effective than pulses at 4 h intervals). Testosterone production stimulated by hCG and supported by 25-hydroxycholesterol was significantly greater than basal in all treatment groups. Testosterone production stimulated by hCG was significantly less than that supported by 25-hydroxycholesterol in anti-GnRH-treated rats given saline or LH pulses, but not in normal serum-treated controls or in anti-GnRH-treated rats given continuous infusions of LH. Differences in the two modes of testosterone production were also apparent in the ratios of substrate-supported to hormone-stimulated production (Table 1).

Activities of the two cytochrome P-450 enzymes, cholesterol side-chain cleavage and $17\alpha\text{-hydroxylase}$, differed among groups in the same pattern as testosterone production supported by 25-hydroxycholesterol, but the decrease in side-chain cleavage activity after anti-GnRH treatment (about 80%) was greater than that in $17\alpha\text{-hydroxylase}$ activity (about 65%) (Fig. 2). Differences in the activities of the P-450 enzymes were accompanied by parallel differences in the contents of immunodetectable enzyme proteins (Fig. 3).

Activity of the non-P-450 enzyme, $3\beta\text{-HSD-isomerase}$, also decreased after 5 days of treatment with anti-GnRH serum (Fig. 4), but not as much as did the activities of the P-450 enzymes (only about 40%). All of the LH treatment regimens prevented this decrease in enzyme activity or increased the activity to values even greater than those in control rats treated with normal serum (Fig. 4). There was no consistent pattern in the effects of the different LH regimens on this enzyme activity.

Fig. 1. Testosterone production: basal (■), stimulated by human chorionic gonadotrophin (□) and supported by 25-hydroxycholesterol (▨) in Leydig cells from rats treated for 5 days with normal serum or with antigonadotrophin-releasing hormone serum and vehicle ('saline') or luteinizing hormone (LH), given as continuous infusions of 12.5 ng per 100 g h⁻¹ (continuous LH) or as 1-min pulses of 25 ng per 100 g every 2 h (2-h LH pulses) or 50 ng per 100 g every 4 h (4-h LH pulses). Data are depicted as mean $\pm$ SEM ($n=6-7$ per group). For a given mode of testosterone production, values with different lower-case letters are significantly different ($P < 0.05$) among treatment groups. Different lower-case letters for different modes of testosterone production within a treatment group do not imply statistically significant differences.

Discussion

Previous studies in our laboratory have shown that maintenance of testosterone-producing capacity stimulated by hormone or second messenger or supported by substrate in Leydig cells can be accomplished with continuous i.v. infusions of exogenous LH as well as with infusions of LH pulses delivered every 2 h during passive immunization against GnRH. Since steroid hormone production is not generally limited by the activities of the enzymes that convert cholesterol to the secreted hormone, however, those results did not prove that maintenance of these enzymes is also independent of the mode of LH administration (pulsatile versus continuous). The present results show that to be the case for at least three of the four enzymes involved in testosterone production from cholesterol in rat Leydig cells: the mitochondrial and microsomal cytochrome P-450 enzymes, cholesterol side-chain cleavage and 17 α -hydroxylase, and the microsomal non-P-450 enzyme 3 β -HSD-isomerase. The activities and immunodetectable contents of both of the cytochrome P-450 enzymes decreased markedly during 5 days of anti-GnRH treatment and these decreases were prevented by all the LH infusion regimens tested. The dependence of both cytochrome P-450 enzymes on LH in this model contrasts with the high constitutive synthesis of P-450_{SCC} in cultured mouse Leydig cells (Anakwe & Payne, 1987; Hales & Payne, 1989). Whether this reflects a difference between species or the presence of an inhibitor *in vivo* (the action of which is suppressed by LH) remains to be determined. The activity of 3 β -HSD-isomerase did not decrease nearly as much during anti-GnRH treatment, but it was increased by concurrent infusion of LH in all of the regimens. The relative lack of dependence of 3 β -HSD-isomerase on LH in this model is in good accord with results from studies in which LH secretion was suppressed by implantation of Silastic capsules containing testosterone and oestradiol (Wing *et al.*, 1985; Keeney *et al.*, 1988).

The last of the enzymes involved in testosterone production, 17-ketosteroid reductase, which catalyses the conversion of androstenedione to testosterone, has received much less attention than

Table 1. Ratio of testosterone production supported by 25-hydroxycholesterol (A) to that stimulated by human chorionic gonadotrophin (B) in Leydig cells from rats treated as described in the legend to Fig. 1

Treatment	A:B
Normal serum	
+ saline	1.17 ± 0.14 ^c
Anti-GnRH serum	
+ saline	2.32 ± 0.31 ^a
+ continuous LH	1.37 ± 0.07 ^{bc}
+ 2h pulses of LH	1.69 ± 0.09 ^{ab}
+ 4h pulses of LH	2.14 ± 0.21 ^a

Results are given as mean ± SEM; values with different superscript letters are significantly different ($P < 0.05$).

Fig. 2. Cholesterol side-chain cleavage (■) and 17α-hydroxylase (□) activities in Leydig cells from rats treated as indicated in Fig. 1. The y axes for the two enzymes are drawn to different scales.

the preceding enzymes. Treatment with hCG *in vivo* has been reported to reduce its activity in rat Leydig cells (O'Shaughnessy & Payne, 1982), suggesting that it is subject to different regulation from the other enzymes and would be worth examining in the model described here.

The differences among LH infusion groups in testosterone production and cytochrome P-450 enzyme activities showed a consistent trend: continuous infusions were more effective than pulses at 2 h intervals and these in turn were more effective than pulses at 4 h intervals. This trend suggests that the frequency of LH exposure is more important than the amplitude of individual exposures in maintaining Leydig cell steroidogenic function. Studies are now in progress to confirm this and to rule out the possibility that an apparent relative inefficacy of low-frequency, high-amplitude LH pulses (in infusion regimens delivering a fixed daily dosage of hormone) is due to a negative effect of the high amplitude (similar to the well-known steroidogenic desensitization caused by supraphysiological doses of LH or hCG) rather than to a lesser positive effect of the low frequency.

Perhaps the most interesting component of the data presented here is the consistent trend in the differences between hCG-stimulated and 25-hydroxycholesterol-supported testosterone

Fig. 3. Autoradiograms of western blots of Leydig cell lysates immunolabelled for (a) P-450_{SCC} and (b) P-450_{17α}. Lysates representing (a) 1×10^5 and (b) 0.6×10^5 Leydig cells were resolved electrophoretically in 10% acrylamide gels, transferred to polyvinylidene fluoride membranes and labelled sequentially with anti-P-450_{SCC} or anti-P-450_{17α} and ^{125}I -protein A. Lanes representing Leydig cells from three rats from each experimental group, which were processed together, are shown.

production among groups. In normal serum-treated control animals, the ability of Leydig cells to produce testosterone from endogenous substrate under the influence of hCG was almost as great as their ability to produce testosterone from exogenous 25-hydroxycholesterol. In anti-GnRH-treated animals given no LH replacement, in contrast, the capacity to produce testosterone from 25-hydroxycholesterol averaged more than twice the capacity to produce testosterone from endo-

Fig. 4. Δ^5 - 3β -hydroxysteroid dehydrogenase- Δ^5 - 4 -isomerase (3β -HSD-isomerase) activity in Leydig cells from rats treated as indicated in Fig. 1.

genous substrate in response to hCG stimulation. The better an LH infusion regimen was at maintaining steroidogenic capacity and cytochrome P-450 enzyme activities, the better it was at maintaining the capacity to respond to hCG stimulation at values near exogenous substrate-supported testosterone production. Such differences and trends were not apparent after LH secretion was suppressed for 5 days more than in the present experiments: after 10 days of passive immunization against GnRH, 25-hydroxycholesterol-supported testosterone production was reduced as severely as was hCG-stimulated production (Gibson-Berry & Chase, 1990). Taken together, the data suggest that some constituent(s) of Leydig cells before cholesterol side-chain cleavage enzyme is (are) more sensitive to withdrawal of LH and deviations from 'optimal' LH exposure than are the side-chain cleavage and subsequent enzymes in the testosterone biosynthetic pathway. LH receptors can probably be ruled out on the basis that ^{125}I -hCG-binding capacity of Leydig cells is not significantly altered by 10 days of anti-GnRH treatment (Gibson-Berry & Chase, 1990). Fragmentary data have suggested that the following might all be subject to long-term regulation by LH: LH receptor-G-protein-adenylate cyclase coupling (Dix *et al.*, 1982; Schumacher *et al.*, 1984); 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Charreau *et al.*, 1981), the rate-limiting enzyme in cholesterol biosynthesis; and sterol carrier protein₂ (van Noort *et al.*, 1986) and steroidogenesis activator polypeptide (Pedersen, 1987), two of the factors implicated in transport of cholesterol to the side-chain cleavage enzyme or association of the substrate with the enzyme's active site in the inner mitochondrial membrane. Systematic studies using the model described here are in progress to determine whether LH receptor-G-protein-adenylate cyclase coupling, cyclic AMP-generating capacity, cholesterol content and mechanisms involved in delivery of cholesterol to cholesterol side-chain cleavage enzyme are subject to long-term regulation by LH and are more dependent on and sensitive to trophic actions of the hormone than are the enzymes involved directly in the synthesis of testosterone from cholesterol.

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