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REGULATION BY DEXAMETHASONE OF THE 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN ADULT RAT LEYDIG CELLS

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Summary—The effect of long-term *in vitro* treatment with dexamethasone, insulin and/or LH on the 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and the testosterone level was examined in cultures of Leydig cells from adult rats. A rapid and simple method for measuring the 3β-HSD activity has been developed, in which the NADH, generated by 3β-HSD, reduced nitroblue tetrazolium to a product with absorption maximum at 560 nm. K_m for the reaction was 8.1 μM and V_{max} was 12.7 nmol/min × mg protein. Addition of 0.1 or 1 μM dexamethasone for 44 h decreased the 3β-HSD activity to 83% and the basal testosterone level to 64% of control value after 22 and 44 h of culture. Addition of 1 nM insulin inhibited the 3β-HSD activity to 90% after 44 h of culture, whereas the testosterone level was increased after 3 h. Addition of 0.1 ng/ml LH did not affect the 3β-HSD activity in Leydig cells from adult rats. Concomitant treatment of the cells with dexamethasone and insulin inhibited the 3β-HSD activity to 74%, indicating an additive effect, whereas no additive effect on the testosterone level was observed. The results demonstrate that the 3β-HSD activity can be measured in a rapid and reliable way by measuring the reduction of nitroblue tetrazolium. Furthermore, the results suggest that dexamethasone acts on 3β-HSD through a mechanism different from that of insulin, as an additive effect was observed.

INTRODUCTION

The synthesis of testosterone from cholesterol in the Leydig cells of testes involves a number of enzymes located in mitochondria and endoplasmic reticulum. Side-chain cleavage enzyme (cytochrome *P*450scc), a mitochondrial enzyme, catalyzes the conversion of cholesterol to pregnenolone. The enzyme, 3β-hydroxysteroid dehydrogenase (3β-HSD), located in the endoplasmic reticulum, catalyzes the dehydrogenation and isomerization of pregnenolone to progesterone. Previous studies demonstrate that the 3β-HSD activity in the testis is limited to the Leydig cells [1, 2]. Cytochrome $P450_{17\alpha}$ catalyzes two reactions, the conversion of progesterone to 17α-hydroxyprogesterone and subsequent cleavage of the two-carbon side-chain to yield androstenedione, the immediate precursor of testosterone. The enzymes involved in testosterone production are affected by hormones. The activities of cytochrome *P*450scc and $P450_{17\alpha}$ are inhibited by dexamethasone as well as by other glucocorticoids [3, 4] and by desensitizing doses of LH [5]. The 3β-HSD activity is inhibited by basic fibroblast growth factor and platelet derived growth factor [6, 7].

The method traditionally used to determine the activity of 3β-HSD measures the conversion of radioactive pregnenolone to progesterone [8]. The method is time-consuming and involves extraction, evaporation, redissolution, and separation of the metabolites by TLC. The spots are cut and counted by a liquid scintillation counter.

This study describes a rapid, simple, and less expensive method for measuring the 3β-HSD activity, based on the well-known staining for 3β-HSD activity in Leydig cells [9]. The method was applied to study the *in vitro* effect of dexamethasone, insulin, and LH on the 3β-HSD activity in Leydig cell cultures.

The data presented here show that addition of dexamethasone or insulin decreased the 3β-HSD activity in long-term cell cultures of Leydig cells from adult rat testis. The effect of the two hormones was additive. The basal

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testosterone level was also decreased by dexamethasone.

EXPERIMENTAL

Materials

Dexamethasone phosphate was from Merck, Sharp & Dohme (Haarlem, The Netherlands) and insulin from Novo-Nordisk (Copenhagen, Denmark). oLH was kindly provided by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Pituitary Program (University of Maryland, School of Medicine, U.S.A.). Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM/F-12), antibiotics (penicillin 10.000 IU/ml and streptomycin 10 mg/ml), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), Earle's balanced salt solution (EBSS), soybean trypsin inhibitor, epiandrosterone, pregnenolone, β-nicotinamide adenine dinucleotide (NAD^+) and testosterone were from Sigma (St Louis, MO, U.S.A.). Percoll was from Pharmacia (Uppsala, Sweden), and collagenase (CLS 1) from Worthington Biochemical Corp. (NJ, U.S.A.). Nitroblue tetrazolium chloride (3,3'-(3,3'-dimethoxy-4,4'-biphenylylen)-bis-[2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride]), sodium dithionite and EDTA were from Merck (Darmstadt, Germany) and nicotinamide was from Rôche (Basel, Switzerland). Dithiothreitol was from Boehringer (Mannheim, Germany), fetal calf serum from Gibco (Paisley, Scotland), and [^{3}H]testosterone ([1,2,6,7-^{3}H]testosterone, 98 Ci/mmol) from Amersham Int. (Amersham, Bucks., England).

Isolation of Leydig cells

Adult male Sprague–Dawley rats (330–430 g) were from Møllegaard Breeding Ctr. (Ll. Skensved, Denmark) or from our own colony. Animals were killed by decapitation, testes removed and placed in DMEM/F-12 containing 10.000 IU/ml penicillin, 10 mg/ml streptomycin, 15 mM HEPES, 0.1% BSA and 1.2 g sodium bicarbonate, pH 7.4. Interstitial cells were obtained by incubating decapsulated testes at 37°C for 30 min in medium containing 0.5 mg/ml collagenase and 0.02% soybean trypsin inhibitor. After filtering the cell suspension through a nylon gauze (pore size 60 μm), and centrifugation at 100 *g* for 10 min, the interstitial cells were further purified on a discontinuous gradient of Percoll in EBSS as described by Sharpe and Fraser [10]. The densities of the gradients were 1.03, 1.05, 1.07, and 1.09. The amount corresponding to four testes was layered on each gradient, and the cells were separated by centrifugation at 1000 *g* for 25 min as described previously [11]. The Leydig cell enriched fractions were collected, washed, and resuspended in medium. The cell suspension contained 75–80% Leydig cells, determined as the proportion of 3β-HSD positive cells of all nucleated cells [9]. The cell density was determined, using a hemacytometer, to be $(4 \pm 0.48) \times 10^7$ cells/ml ($n = 11$, mean $\pm$ SEM) and the protein content was found to be 6.4 $\pm$ 0.8 mg protein/ml. The viability of the cells was determined to be approx. 95% by the trypan blue exclusion method.

Culture and incubation of Leydig cells

The cell suspensions were plated in 35 mm Costar wells (approx. 1×10^7 cells/well). The culture medium was DMEM/F-12 supplied with 0.1 or 1 μM dexamethasone, 1 nM insulin and/or 0.1 ng/ml LH, and for the first 22 h the culture medium contained 1% fetal calf serum. The culture medium was changed after 22 h. The cells were cultured at 34°C in a 5% CO_2–air atmosphere. The analyses were performed after 3, 22 and 44 h of culture. The cellular response of 10 ng/ml LH for 2 h was estimated after 22 h of culture. Before changing to medium containing 10 ng/ml LH the cells were washed twice and incubated for 30 min in medium in order to remove the steroids and hormones from the cells.

Determination of the 3β-HSD activity

The Leydig cells were harvested in 0.1 M phosphate buffer containing 1 mM EDTA and 1 mM dithiotreitol, kept at −80°C, and analyzed within 2 days. The cell preparation was thawed on a water bath at 37°C and sonicated. 300 μl (200–400 μg protein) homogenate was mixed with a reagent consisting of 0.1 mM epiandrosterone dissolved in DMSO (1%), 0.2 mM nitroblue tetrazolium, 1.4 mM nicotinamide and 0.5 mM NAD^+ in 0.1 M sodium phosphate buffer, pH 7.4, to a final volume of 1 ml. Immediately after having mixed the cell preparation and the reagent at 36–38°C, the change of absorbance was measured spectrophotometrically (Uvicon spectrophotometer 930) at 560 nm for 10 min. The initial rate was calculated, and related to the protein concentration, determined after the method of Lowry *et al.* [12].

Radioimmunoassay (RIA)

The testosterone concentration in media was determined by RIA. The antibody was kindly provided by Dr Paul Bennett (Hormone Department, Statens Serum Institut, Copenhagen) [13]. The testosterone level was related to the protein concentration.

Statistical analysis

All the experiments were made in duplicate. The number of experiments is indicated in the figures and tables. Student's t-test for paired data was used to evaluate the results. $P < 0.05$ was considered statistically significant.

RESULTS

Estimation of the 3β-HSD activity

The present method for determination of the 3β-HSD activity was based on the method for identification of Leydig cells [9]. Epiandrosterone, the substrate of 3β-HSD, was dehydrogenated to androstandione. In the same reaction NAD^+, the coenzyme of 3β-HSD, was reduced to NADH, which in turn reduced nitroblue tetrazolium to a product with absorption maximum at 560 nm.

The presence of NADPH cytochrome c reductase activity was necessary for the reduction of nitroblue tetrazolium, but experiments showed that this enzyme was present in the cell homogenate, and that no further addition was needed for the reaction to take place (data not shown).

The molar extinction coefficient of reduced nitroblue tetrazolium was determined to be $1.5 \times 10^4\ M^{-1}\ cm^{-1}$. The reduced nitroblue tetrazolium precipitated in the reagent medium at concentrations larger than 40 μM, but the concentration in the initial reaction was far below this value (data not shown).

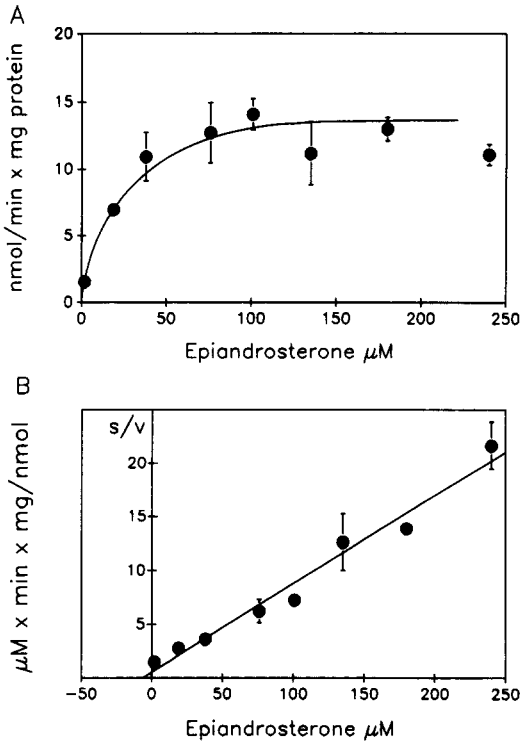

Fig. 1. (A) Michaelis–Menten plot of the 3β-HSD activity. (B) Hanes plot of the 3β-HSD activity. The experiments were made on a Leydig cell suspension, and the substrate used was epiandrosterone. The results represent the mean ± range of duplicate determinations from one typical experiment of four.

The 3β-HSD activity was proportional to a protein concentration up to at least 3.5 mg protein/ml Leydig cell suspension (data not shown).

The Michaelis–Menten and Hanes plots of the 3β-HSD activity are shown in Fig. 1. Kinetic parameters for epiandrosterone as substrate were determined from the initial rates

Table 1. Effects on the 3β-HSD activity of dexamethasone, insulin and LH alone or in different combinations

Dex. μM	Ins. nM	LH ng/ml	Hours of culture (nmol/min × mg protein) 3	22	44
—	—	—	5.07 ± 0.33 (9)	4.77 ± 0.18 (9)	4.57 ± 0.23 (9)
0.1	—	—	5.18 ± 0.25 (9)	4.56 ± 0.23 (9)	3.82 ± 0.33 (8)[a,b]
1.0	—	—	5.44 ± 0.18 (6)	4.65 ± 0.39 (6)	3.70 ± 0.20 (6)[a]
—	1.0	—	5.23 ± 0.28 (6)	4.63 ± 0.15 (6)	4.17 ± 0.20 (6)[a,b]
—	—	0.1	4.72 ± 0.44 (5)	5.09 ± 0.25 (5)	4.51 ± 0.13 (5)
0.1	1.0	—	5.59 ± 0.49 (6)	4.42 ± 0.24 (6)	3.43 ± 0.46 (6)[a]
0.1	—	0.1	5.31 ± 0.45 (4)	5.12 ± 0.23 (4)	3.62 ± 0.25 (4)[a]
0.1	1.0	0.1	5.14 ± 0.37 (4)	4.84 ± 0.31 (4)	3.84 ± 0.23 (4)[a]

Leydig cells were obtained from adult male rats cultured up to 44 h in DMEM/F-12 without preincubation. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses. Dex, Ins and LH indicate dexamethasone, insulin and luteinizing hormone, respectively.

[a]Indicates a value significantly different from control.

[b]Indicates a value significantly different from cultures with both dexamethasone and insulin.

using the weighted and non-linear regression method described by Wilkinson [14]. The K_m was $8.1 \pm 3.5\,\mu M$ and V_{max} was 12.7 ± 0.6 nmol/min × mg protein ($n = 4$, mean ± SEM). Pregnenolone was also tested as substrate in the analysis, but precipitated in the reagent at concentrations larger than $50\,\mu M$, making it unsuitable as substrate. K_m was estimated to be larger than $40\,\mu M$ and V_{max} to be about 5 nmol/min × mg protein using pregnenolone concentrations of 20 and $50\,\mu M$.

Effect of hormones on the 3β-HSD activity

The effect on the 3β-HSD activity in Leydig cell cultures treated with 0.1 or $1\,\mu M$ dexamethasone, 1 nM insulin or 0.1 ng/ml LH alone or in different combinations was investigated after 3, 22 and 44 h of culture (Table 1). After 44 h of treatment dexamethasone and insulin significantly decreased the 3β-HSD activity to 83 and 90% of control value, respectively. No difference was observed for the two concentrations of dexamethasone. Concomitant treatment with $0.1\,\mu M$ dexamethasone and 1 nM insulin significantly decreased the 3β-HSD activity to 74%, indicating an additive effect of the two hormones. LH (0.1 ng/ml) had no effect on the 3β-HSD activity. Addition of both $0.1\,\mu M$ dexamethasone and 0.1 ng/ml LH showed that LH could not abolish the inhibitory effect of dexamethasone. If all three hormones were present, the decrease was comparable to the inhibition observed with dexamethasone only.

Effect of hormones on the basal testosterone level

The effect on testosterone levels was analyzed after addition of 0.1 or $1\,\mu M$ dexamethasone, 1 nM insulin and/or 0.1 ng/ml LH after 3, 22 and 44 h of culture (Table 2).

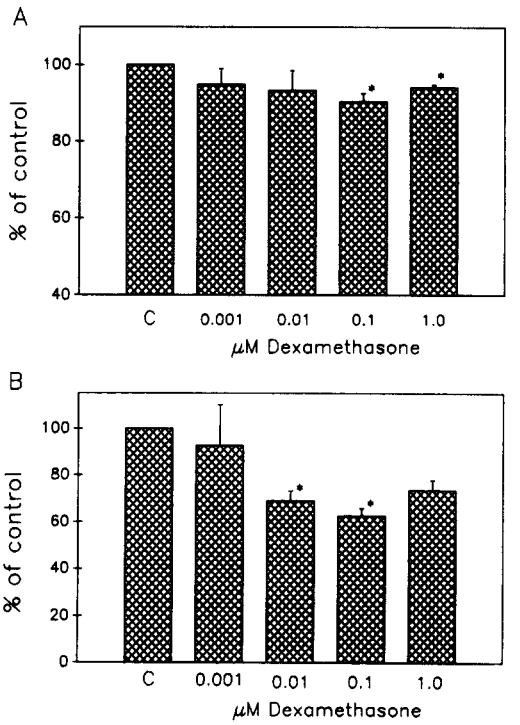

Fig. 2. (A) Dose–effect curve of dexamethasone on the 3β-HSD activity as percent of control C. The control value corresponds to 4.43 ± 0.88 nmol/min × mg protein. (B) Dose–effect curve of dexamethasone on the testosterone level as percent of control C. The control value corresponds to 531 ± 161 ng/mg protein. Each value is the mean ± SEM of three separate experiments performed in duplicate on Leydig cells culture for 44 h with or without dexamethasone.
*Indicates a value significantly different from control.

Addition of 0.1 or $1\,\mu M$ dexamethasone decreased the basal testosterone level to approx. 64% of control value after 22 and 44 h of culture with no significant difference observed between the two concentrations. After 3 h of culture 1 nM insulin increased the testosterone level to 150%, while no effect was observed after 22 and 44 h of culture. Addition of 0.1 ng/ml

Table 2. Effects on the testosterone level of dexamethasone, insulin and LH alone or in different combinations

Dex. μM	Ins. nM	LH ng/ml	Hours of culture (ng testosterone/mg protein) 3 h/3 h*	22 h/22 h*	44 h/22 h*
—	—	—	150 ± 44 (4)	325 ± 52 (4)	418 ± 90 (6)
0.1	—	—	131 ± 23 (4)	215 ± 27 (4)[a]	285 ± 57 (6)[a]
1.0	—	—	135 ± 29 (4)	191 ± 23 (4)[a]	261 ± 60 (6)[a]
—	1.0	—	229 ± 37 (4)[a]	317 ± 17 (4)	406 ± 85 (6)
—	—	0.1	436 ± 137 (3)[a]	527 ± 96 (4)[a]	630 ± 166 (6)
0.1	1.0	—	163 ± 31 (4)	220 ± 11 (4)	300 ± 85 (6)[a]
0.1	—	0.1	310 ± 75 (4)	542 ± 114 (4)	716 ± 126 (6)[a]
0.1	1.0	0.1	289 ± 30 (4)[a]	488 ± 124 (4)	792 ± 31 (6)[a]

Leydig cells were obtained from adult male rats cultured up to 44 h in DMEM/F-12 without preincubation. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses. Dex, Ins and LH indicate dexamethasone, insulin and luteinizing hormone, respectively.
[a]Indicates a value significantly different from control.
*Indicates hours of culture/hours of testosterone production.

LH increased the testosterone level to 290, 160 and 150% after 3, 22 and 44 h, respectively.

Concomitant treatment with 0.1 μM dexamethasone and 1 nM insulin abolished the stimulatory effect of insulin after 3 h of culture. After 22 h the testosterone level was decreased to approx. 70% with no further decrease after 44 h. Combination of 0.1 μM dexamethasone and 0.1 ng/ml LH increased the testosterone level to 207, 167 and 171% after 3, 22 and 44 h of culture, respectively. The data did not differ significantly from data obtained with cultures treated with LH only. The same was observed when all three hormones were present.

Dose–response curves

Dexamethasone inhibited the 3β-HSD activity in a dose-dependent manner, with the largest inhibition observed for 0.1 μM dexamethasone [Fig. 2(A)]. Furthermore, the testosterone level was decreased in a dose-dependent manner, with the largest decline at concentrations of 0.1 and 1 μM [Fig. 2(B)].

LH (0.1, 1.0, 5.0, and 10 ng/ml) had no effect on the 3β-HSD activity when treated for up to 44 h (data not shown). The testosterone level was increased in a dose-dependent manner (data not shown).

Stimulation with 10 ng/ml LH

Stimulation of Leydig cells, pretreated with hormones for 22 h, with 10 ng/ml LH present for 2 h, had no effect on the 3β-HSD activity (data not shown). The basal testosterone level was increased approx. 20 times by 10 ng/ml LH for 2 h (Table 3). The testosterone levels of cultures pretreated with hormones did not deviate significantly from control cells after 2 h of culture with or without 10 ng/ml LH (Table 3).

Table 3. Effects on the testosterone production of preincubation with dexamethasone, insulin and/or LH for 22 h, followed by incubation with or without 10 ng/ml LH for 2 h

Dex. μM	Ins. nM	LH ng/ml	(ng testosterone/mg protein × 2 h) −LH	+LH
—	—	—	21.8 ± 4.2 (4)	428 ± 63 (6)
0.1	—	—	17.7 ± 6.7 (4)	509 ± 92 (6)
1.0	—	—	18.4 ± 7.9 (4)	548 ± 109 (6)
—	1.0	—	24.5 ± 10.9 (4)	401 ± 118 (6)
—	—	0.1	23.5 ± 14.4 (4)	432 ± 114 (6)
0.1	1.0	—	13.0 ± 6.5 (4)	518 ± 83 (6)
0.1	—	0.1	15.4 ± 5.9 (4)	536 ± 113 (6)
0.1	1.0	0.1	18.2 ± 8.5 (4)	554 ± 111 (6)

Leydig cells were obtained from adult male rats cultured in DMEM/F-12. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses. Dex, Ins and LH indicate dexamethasone, insulin and luteinizing hormone, respectively.

DISCUSSION

For many years 3β-HSD activity has been measured in different cell types, including Leydig cells. The method in general use involves labeled pregnenolone [8, 15] or dehydroepiandrosterone [16]. It seems to be good and reliable, the only disadvantages being the time-consumption and the costs. The advantage of the present method is that it is cheaper and much more rapid. In another method the NADH, arising during the 3β-HSD-catalyzed dehydrogenation, was measured spectrophotometrically at 340 nm [1]. The present method differs in the addition of nitroblue tetrazolium, which is reduced by NADH to a product measured spectrophotometrically at 560 nm. This improves the sensitivity of the test as the molar extinction coefficient of NADH is 6220 M^{-1} cm^{-1}, whereas it is 1.5 × 10^4 M^{-1} cm^{-1} for reduced nitroblue tetrazolium.

In this study, epiandrosterone is used as substrate for 3β-HSD, and a K_m of 8.1 μM was determined. Using pregnenolone as substrate, K_m was found to be 40 μM, in agreement with a reported value of 33 μM for 3β-HSD purified from rat testis [17]. The present method was used to investigate the effect of different hormones on testicular 3β-HSD activity in cultured Leydig cells.

The presence of glucocorticoid receptors in interstitial cells of rat testis was first demonstrated by Evain *et al.* [18]. The majority of these receptors was located in the Leydig cells [19]. Glucocorticoids lowered the LH receptor content of gonadotropin stimulated Leydig cells [20], and inhibited the steroidogenic enzyme, cytochrome $P450_{17\alpha}$ [3]. In mouse interstitial cells glucocorticoids decreased the constitutive and cAMP-induced synthesis of cytochrome P450scc protein and accumulation of cytochrome P450scc mRNA [4]. On the other hand, in MA-10 tumor Leydig cells glucocorticoids increased the rate of cytochrome P450scc synthesis and the amount of mRNA [21].

This study shows that dexamethasone, a synthetic glucocorticoid, decreased the 3β-HSD activity in a dose-dependent manner. In agreement with this Payne *et al.* [22] have recently reported that dexamethasone reduced the basal 3β-HSD mRNA content of mouse Leydig cells. From this and other studies dealing with steroidogenic enzyme activities [3, 4] it may be concluded that most of the enzymes involved in steroidogenesis are down-regulated by glucocorticoids.

Dexamethasone decreased the basal testosterone level after an incubation period of 22 h, and the inhibition persisted throughout the rest of the culture period. Most of the studies concerning the effect of glucocorticoids on the testosterone production have dealt with the hCG/LH-stimulated testosterone synthesis [3, 18, 20, 23]. Glucocorticoids suppress the hCG/LH-induced testosterone production [3, 18, 20, 23], and the inhibition is dose-dependent [3, 20]. This is in contrast to the present results, which show that dexamethasone did not affect the LH-induced (0.1 ng/ml) testosterone level. The use of a submaximal dose of LH in this study as against a maximal dose in the other studies [3, 18, 20, 23] may explain the disagreement.

LH (0.1–10 ng/ml) did not affect the activity of 3β-HSD. This is in agreement with a recent report by Keeney *et al.* [26], who found that the 3β-HSD activity of adult rats was unaffected by treatment with LH. The 3β-HSD activity of Leydig cells from rats about 25 days old was stimulated *in vitro* by hCG/LH in long term cultures [6, 15, 24], whereas the effect was only small [25] or absent in older rats [15, 26]. The same results were reported, when the rats were treated *in vivo* [27, 28]. The 3β-HSD activity of hypophysectomized rats increased when treated with hCG/LH [8, 29–31]. Stimulation of the 3β-HSD activity seems to depend on the age of the rats as well as on the culture conditions. Furthermore, this study shows that LH (0.1 ng/ml) did not affect the inhibition of dexamethasone exerted on the 3β-HSD activity after 44 h of treatment.

Addition of 0.1–10 ng/ml LH increased the testosterone level after 3, 22 and 44 h of culture with the largest increment after 3 h. This is in agreement with other reports showing a large initial hCG/LH-induced stimulation of the testosterone response, whereupon the response declined with time due to desensitization [32].

Specific insulin receptors are also present on Leydig cells [33]. Animals suffering from diabetes have reduced gonadotropin receptor content, which is restored to normal values after administration of insulin [34]. The present study shows that 1 nM insulin significantly decreased the 3β-HSD activity after a treatment period of 44 h. In immature rats insulin was reported to enhance the 3β-HSD activity after treatment for 6 days [15]. Thus, the insulin-induced 3β-HSD activity may be enhanced in immature rats [15], whereas it may be decreased in mature rats, as suggested by our study.

It was found that 1 nM insulin increased the testosterone level after 3 h of culture, however, no effect was observed after 22 and 44 h. This is in agreement with reports showing that insulin augmented basal testosterone production in short term incubations [35], whereas no effect was observed after 2–10 days of culture [36]. Concomitant treatment of Leydig cell cultures with dexamethasone and insulin for 44 h resulted in additive effects of the hormones on the 3β-HSD activity, indicating that the two hormones acted on 3β-HSD through different mechanisms. The additive effect on 3β-HSD was not reflected in the testosterone level.

Simultaneous addition of dexamethasone, insulin and LH to Leydig cell cultures resulted in a decrease in 3β-HSD activity in the same order of magnitude as that seen for cultures treated with dexamethasone only. The inhibitory effect caused by insulin may be abolished by LH, although not significantly. When all three hormones were added no effect on the testosterone level was observed compared to the response caused by LH alone.

Pretreatment of the cells with dexamethasone for 22 h, following incubation with or without 10 ng/ml LH for 2 h, showed no significant inhibition of the LH-stimulated testosterone synthesis. This suggests that inhibition with dexamethasone was reversible, as reported by Welsh *et al.* [3].

The present results demonstrate that the 3β-HSD activity can be measured in a fast and reliable way by measuring the reduction of nitroblue tetrazolium. Furthermore, it is found that the 3β-HSD activity is inhibited by dexamethasone and insulin, and that the effect of the two hormones is additive, indicating that dexamethasone acts through a mechanism different from that of insulin.

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