Pergamon

0960-0760(95)00058-5

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 1/2, pp. 75–81, 1995

0960-0760/95 $9.50 + 0.00

Effects of Dexamethasone on Steroidogenesis in Leydig Cells from Rats of Different Ages

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The effects of 0.1 μM dexamethasone on cytochrome *P*450 content, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, and basal and LH-induced testosterone production of Leydig cells from rats 3, 5, 7 and 10 weeks old were examined. The cytochrome *P*450 content of Leydig cells from rats 3 weeks old was increased by treatment with dexamethasone for 22 h, while 3β-HSD activity was decreased. The cytochrome *P*450 content of Leydig cells from rats 5 weeks old was increased after 3 and 22 h of culture, while 3β-HSD activity was decreased after 22 and 44 h of treatment. The cytochrome *P*450 content of rats 7 weeks old was increased after 3 h of culture, while 3β-HSD activity was decreased after 22 and 44 h of culture. Leydig cells from rats 10 weeks old showed increased cytochrome *P*450 content upon dexamethasone treatment after 3 h. The activity of 3β-HSD was decreased after 44 h of treatment. In Leydig cells from rats 3 and 5 weeks old, dexamethasone decreased basal testosterone production after 22 h of treatment, but not after 44 h, and did not affect LH-induced testosterone production. Leydig cells from rats 7 weeks old showed decreased basal and LH-induced testosterone production, when treated with dexamethasone for 22 and 44 h. Basal testosterone production was unaffected by dexamethasone in rats 10 weeks old, while LH-induced testosterone production was decreased after 44 h of treatment. The effect of dexamethasone on testosterone secretion changed during development, as a transient, early effect on basal testosterone secretion was observed in Leydig cells from prepubertal and pubertal rats. These data suggest that dexamethasone affects Leydig cells differently, depending on the age of the rat, the older rats being more sensitive than the younger rats.

J. Steroid Biochem. Molec. Biol., Vol. 54, No. 1/2, pp. 75–81, 1995

INTRODUCTION

Induction of cytochrome *P*450 enzymes in hepatocytes is an area of research which has obtained much attention during the last decades. However, extrahepatic tissues contain cytochrome *P*450 enzymes which are inducible by xenobiotics as well.

Leydig cells contain two cytochrome *P*450 enzymes involved in testosterone production: cytochrome *P*450scc (CYP11A1) [1] which catalyzes the conversion of cholesterol to pregnenolone; and cytochrome *P*450 17α/C_{17-20} lyase (CYP17) [1] which is responsible for two reactions, the hydroxylation of progesterone to 17α-hydroxyprogesterone, and the subsequent conversion to androstenedione. In Leydig cells of adult animals, cytochrome *P*450 enzymes also catalyze the hydroxylation of testosterone; 7α- and 6α-testosterone hydroxylases being dominant [2]. In this study the cytochrome *P*450 content was measured by spectral analysis, which is monitoring a wide variety of cytochrome *P*450 enzymes. This method was developed as a tool to screen xenobiotics for possible effects on Leydig cells. However, an effect could be overlooked, as some cytochrome *P*450 isoforms might be induced and others inhibited. It has previously been reported that dexamethasone decreased cytochrome *P*450s involved in testosterone synthesis [3]. However, the spectral analysis in this study showed that some cytochrome *P*450 isoforms were induced by dexamethasone as well.

In order to study the effect of exogenously added factors on Leydig cell steroidogenic activity, the hourly rate of steroid production in cultures of Leydig cells must maintain a constant level [4]. A steroid production similar or close to the *in vivo* production can only be maintained if the cytochrome *P*450 enzymes catalyzing the different reactions in testosterone synthesis are maintained.

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Received 22 Sep. 1994; accepted 1 Feb. 1995.

The aim of this study was to examine the effects of exogenously added factors on steroidogenesis in cultures of Leydig cells from rats of different developmental stages. In an attempt to maintain the cytochrome *P*450 content in cultures of Leydig cells, the cells were treated with dexamethasone and metyrapone, substances known to induce and stabilize cytochrome *P*450 in cultures of hepatocytes [5–7]. The total amount of cytochrome *P*450, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and basal, as well as LH-induced, testosterone secretion were analyzed.

MATERIALS AND METHODS

Materials

Dexamethasone phosphate was from Merck, Sharp & Dohme (Haarlem, The Netherlands). oLH was kindly provided by the 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, soybean trypsin inhibitor, epiandrosterone, β-nicotinamide adenine dinucleotide (NAD^+), testosterone, Triton X-100, and metyrapone [2-methyl-1,2-di-(3-pyridyl)propane-1-one] 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, EDTA and glycerol were from Merck (Darmstadt, Germany) and nicotinamide was from Rôche (Basel, Switzerland). Dithiothreitol was from Boehringer (Mannheim, Germany), foetal calf serum from Gibco (Paisley, Scotland, U.K.), and [^{3}H]testosterone (1,2,6,7-[^{3}H]testosterone, 98 Ci/mmol) and testosterone/dihydrotestosterone ^{3}H-assay system from Amersham International (Amersham, Buckinghamshire, U.K.). Carbon monoxide was from Aga A/S (Copenhagen, Denmark).

Isolation and culture of Leydig cells

Leydig cells were from male Sprague–Dawley rats 3, 5, 7 or 10 weeks old, weighing 54 ± 15 (5), 144 ± 18 (5), 215 ± 38 (6) and 345 ± 24 g (5), respectively (mean ± SD (*n*)). The rats were from our own colony. Leydig cells were isolated on Percoll gradients, as previously described [8]. The cell suspensions from rats 3, 5, 7 and 10 weeks old contained approx. 90, 88, 85 and 82% Leydig cells, respectively, determined as the proportion of 3β-HSD positive cells of all nucleated cells [9]. The cell viability was approx. 95%, as determined by the trypan blue exclusion method.

The cells were suspended 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, with or without 0.1 μM dexamethasone or 0.5 mM metyrapone. The cell suspensions were plated in 35 mm Costar wells with approx. 3×10^6 cells/well, and cultured for 3, 22 or 44 h at 34°C in a 5% CO_2–95% air atmosphere. Dexamethasone or metyrapone were added to the culture medium at the time of plating of the cells. The cellular response of 10 ng/ml LH with and without dexamethasone or metyrapone for 2 h was estimated after 22 and 44 h of culture. Before changing to medium containing LH, the cells were washed twice and incubated for 30 min in medium with and without dexamethasone or metyrapone, in order to remove the steroids from the cells.

Determination of the 3β-HSD activity

3β-HSD activity was determined as previously described [10]. Briefly, the cell preparation was sonicated, and 300 μl (200–400 μg protein) of 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 increase in absorbance, due to reduction of nitroblue tetrazolium ($\epsilon = 1.5 \times 10^4\ M^{-1}\ cm^{-1}$), was measured spectrophotometrically (Uvicon spectrophotometer 930) at 560 nm. The initial rate was calculated, and related to the protein concentration, determined after the method of Lowry *et al.* [11]. Dexamethasone did not affect NADPH cytochrome c reductase, necessary in determination of 3β-HSD activity, in any of the ages tested [10].

Determination of the cytochrome P450 content

Leydig cells were harvested in 200 μl phosphate buffer containing 0.8 mM EDTA, 0.8 mM DTT, 20% glycerol and 2% Triton X-100, pH 7.4, and immediately frozen in liquid nitrogen. The cell homogenate was transferred to a micro precision cuvette and the sodium dithionite-difference spectrum was determined by the method of Omura and Sato [12], modified by Matsubara *et al.* [13]. Cells from parallel dishes were harvested for protein measurement [11]. Determinations were made after 3, 22 and 44 h of culture.

Radioimmunoassay (RIA)

The testosterone concentration in media was determined by RIA. The antibody was kindly provided by Dr Paul Bennett, Hormone Department, Statens Serum Institute, DK-2300 Copenhagen [14]. The

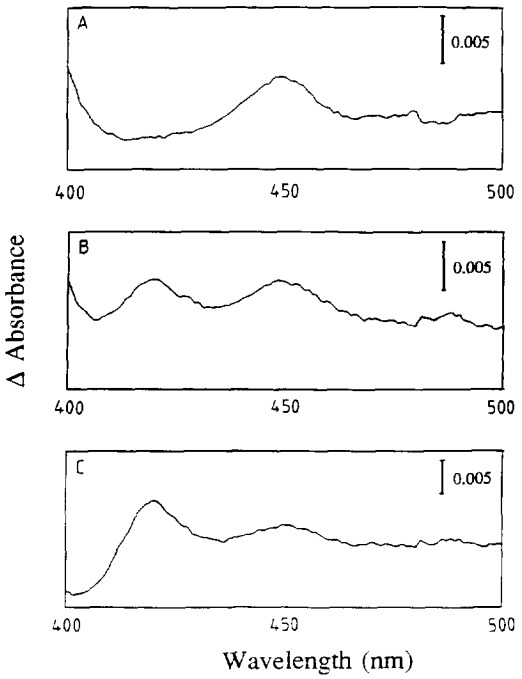

Fig. 1. Dithionite-difference spectra of cytochrome *P*450 in Leydig cells from rats 5 weeks old. (A) Immediately after isolation. (B) After 3 h of culture. (C) After 22 h of culture.

testosterone level was related to the protein concentration [11].

The dihydrotestosterone concentration in media was determined by RIA after oxidative destruction of testosterone followed by ether extraction of dihydrotestosterone using the assay system from Amersham.

Statistical analysis

All the experiments were made in duplicate. The number of preparations is indicated in the Figures and Tables. Multiway analysis of variance and Student's *t*-test for single and paired data were used to evaluate the results. The statistics were performed on the effects and expressed in percentages. $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of cytochrome P450 content

Measurement of cytochrome *P*450 content in hepatocytes is a widely applied method for determination of induction by xenobiotics. The amount of cytochrome *P*450 in Leydig cells, however, is much smaller than in hepatocytes, giving difficulties in measuring the content in Leydig cells. We have overcome these difficulties by using a concentrated cell homogenate, a microcuvette and recording the dithionite-difference spectrum instead of the carbon monoxide-difference spectrum (Fig. 1). The carbon monoxide-difference spectrum showed a disturbing negative peak at 430 nm, possibly originating from cytochrome a_3 [15]. In the dithionite-difference spectrum a peak at 420 nm developed after 22 h of culture, probably due to the breakdown of cytochrome *P*450 to its inactive form, cytochrome *P*420. This peak, in most incidences, did not disturb the peak at 450 nm, however, the amount of cytochrome *P*450 might have been slightly underestimated.

Effect of dexamethasone on cytochrome P450 content

The total amount of cytochrome *P*450 in Leydig cells from rats 3, 5, 7, and 10 weeks old was determined after 3, 22, and 44 h of culture (Table 1). The cytochrome *P*450 content of Leydig cells decreased during culture, the decrease being independent of the age of the rats. During the first 3 h of culture, cytochrome *P*450 content decreased by approx. 35%. (Freshly isolated cells from 7-week-old rats contained 65 ± 4 pmol cytochrome *P*450/mg protein and after 3 h of culture 43 ± 10 pmol/mg protein, this value was not different from that in Table 1 (mean ± SEM, $n = 6$).) Addition of dexamethasone to the culture medium

Table 1. Effect of dexamethasone on the cytochrome P450 content

	Hours of culture			
	3		22	
Age (weeks)	Control (pmol/mg protein)	Dexamethasone (% of control*)	Control (pmol/mg protein)	Dexamethasone (% of control*)
3	73 ± 19 (4)	110 ± 37	15 ± 7 (3)	219 ± 13†
5	96 ± 9 (5)	138 ± 10†	46 ± 9 (5)	143 ± 8†
7	55 ± 15 (6)	132 ± 10†	38 ± 16 (4)	185 ± 50
10	105 ± 33 (9)	163 ± 16†	ND	—

Leydig cells were obtained from rats of different ages, and cultured in 3 or 22 h in DMEM/F-12 with the addition of 0.1 μM dexamethasone. The cytochrome *P*450 content was measured by recording of the dithionite-difference spectrum. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses. ND, not detectable. Treatment with dexamethasone for 22 h had a significantly larger effect when the % of control were compared to 3 h of treatment.

*% of control after treatment with 0.1 μM dexamethasone for 3 or 22 h.

†Indicates a significant value.

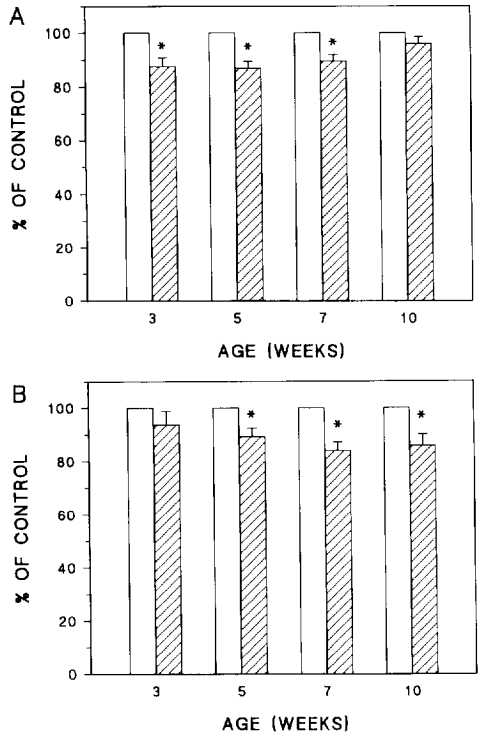

Fig. 2. Effect of 0.1 μM dexamethasone on the 3β-hydroxysteroid dehydrogenase activity in Leydig cells from rats of different ages. Open bars indicate control cells, hatch bars indicate dexamethasone treated cells. (A) After 22 h of treatment. The 3β-HSD activity for untreated rats 3, 5, 7, and 10 weeks old after 22 h of culture were 6.32 ± 1.00 (5), 8.56 ± 1.18 (6), 5.70 ± 0.91 (4), and 4.73 ± 0.23 (9) nmol/(min × mg protein) respectively. (B) After 44 h of treatment. The 3β-HSD activity for untreated rats 3, 5, 7, and 10 weeks old after 44 h of culture were 5.44 ± 0.47 (5), 7.80 ± 0.68 (6), 5.19 ± 0.65 (7), and 5.45 ± 0.64 (9) nmol/(min × mg protein) respectively. Leydig cells were cultured as described under Methods. Each value is the mean ± SEM of 4–9 separate preparations.

significantly increased the cytochrome *P*450 content after 3 h of culture (Table 1) in Leydig cells from rats 5, 7, and 10 weeks old. After 22 h of treatment (Table 1) the cytochrome *P*450 content was significantly elevated in rats 3 and 5 weeks old. The cytochrome *P*450 content of cultures from 7-week-old rats was increased to 185% (not significant), whereas in cultures from rats 10 weeks old, cytochrome *P*450 was not detectable neither in the absence nor in the presence of dexamethasone. In cultures kept for 44 h, there was no detectable cytochrome *P*450 in any of the 4 groups (data not shown). Analysis of variance showed that the effect of dexamethasone was significantly larger after 22 h of treatment than after 3 h.

Effect of dexamethasone on 3β-HSD activity

The 3β-HSD activity in Leydig cells after 22 h and 44 h of incubation was largest in cultures from rats 5 weeks old (legend to Fig. 2).

The effect of dexamethasone on 3β-HSD activity from rats 3, 5, 7, and 10 weeks old was investigated after 3, 22, and 44 h of culture. After 22 h of treatment with dexamethasone the 3β-HSD activities of cultures from rats 3, 5, and 7 weeks old [Fig. 2 (A)] were significantly inhibited to 87, 88 and 87% of control values, respectively, whereas the 3β-HSD activity of rats 10 weeks old was not significantly decreased. The inhibition persisted throughout the 44 h of culture [Fig. 2(B)] (97, 89, 86 and 86% of control values, for 3, 5, 7, and 10-week-old rats, respectively), although the effect observed in cultures from rats 3 weeks old was not significant.

Effect of dexamethasone on the testosterone secretion

The testosterone secretion of Leydig cells in culture increased with the age of the rat, reaching a maximum at the 7th week, whereupon it significantly decreased (approx. 60%) between the 7th and 10th week of age (Table 2).

The effect of dexamethasone on basal and LH-stimulated testosterone secretion was analyzed after 22 and 44 h of culture (Tables 2 and 3). Dexamethasone significantly decreased the basal testosterone secretion of Leydig cells from rats 3, 5, and 7 weeks old after 22 h of culture, the decrease persisting for 44 h only in rats 7 weeks old. On the other hand, LH-stimulated testosterone production was only significantly decreased by dexamethasone after 22 h of culture in 7-week-old rats.

Table 2. Effect of dexamethasone on the testosterone level after 22 h of culture

	−LH		+LH	
Age (weeks)	Control (ng/mg protein × 2 h)	Dexamethasone (% of control*)	Control (ng/mg protein × 2 h)	Dexamethasone (% of control*)
3	2.5 ± 0.2 (5)	38 ± 7†	12 ± 3 (7)	94 ± 6
5	12 ± 2 (7)	41 ± 5†	186 ± 30 (7)	93 ± 10
7	30 ± 8 (7)	34 ± 6†	614 ± 153 (7)	77 ± 4†
10	8 ± 2 (7)	74 ± 20	224 ± 47 (7)	113 ± 34

Leydig cells were obtained from rats of different ages, and cultured for 22 h in DMEM/F-12. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses.

*% of control after treatment with 0.1 μM dexamethasone for 22 h.

†Indicates a significant decrease.

Table 3. Effect of dexamethasone on the testosterone level after 44 h of culture

Age (weeks)	−LH Control (ng/mg protein × 2 h)	−LH Dexamethasone (% of control*)	+LH Control (ng/mg protein × 2 h)	+LH Dexamethasone (% of control*)
3	0.6 ± 0.1 (5)	85 ± 19	2.2 ± 0.3 (4)	79 ± 10
5	2 ± 0.7 (4)	57 ± 14	114 ± 18 (5)	100 ± 14
7	6 ± 1.5 (5)	49 ± 6†	223 ± 40 (5)	71 ± 4†
10	4 ± 1.4 (4)	48 ± 21	111 ± 11 (4)	47 ± 10†

Leydig cells were obtained from rats of different ages, and cultured in 44 h in DMEM/F-12. Each value represents the mean ± SEM, and the number of separate preparations each made in duplicate is indicated in parentheses.

*% of control after treatment with 0.1 μM dexamethasone for 44 h.

†Indicates a significant decrease.

After 44 h of culture a decrease was observed in both 7- and 10-week-old rats.

The effect of dexamethasone on dihydrotestosterone concentration was determined in a single experiment. Dexamethasone decreased the secretion of dihydrotestosterone in the same pattern as for testosterone (data not shown), with rats 3 and 5 weeks old producing more dihydrotestosterone than testosterone, as previously reported [16].

Effect of metyrapone

Metyrapone showed no stabilizing effect on the cytochrome *P*450 content in cultures from any of the ages tested (data not shown). Furthermore, no effect of metyrapone was observed on 3β-HSD activity and basal testosterone production, whereas LH-stimulated testosterone production was inhibited to the level of basal testosterone production (data not shown).

DISCUSSION

In this study a model was developed to screen induction by xenobiotics of cytochrome *P*450 in rat Leydig cells by determination of the total amount of cytochrome *P*450. It has been reported that measuring extrahepatic cytochrome *P*450 was associated with difficulties [17]. The measurement in this study was performed by means of the dithionite-difference spectrum [2, 13], giving reproducible results. The cytochrome *P*450 content of Leydig cells decreased during culture, whereas the peak at 420 nm increased, indicating that cytochrome *P*450 was labile, which was also observed in cultures of hepatocytes [6]. Whether this was a slowly progressive event or a fast degradation associated with harvesting of the cells, was not clear. It might have been a mixture of both events, because, in cultures kept for 44 h, we were unable to detect any cytochrome *P*450, but the Leydig cells still were capable of synthesizing testosterone, indicating that not all cytochrome *P*450 was lost.

The ability of Leydig cells to synthesize testosterone decreased with time of culture, as indicated by the results of us as well as others [18]. The activity of CYP17 was diminished during culture as well [19]. As an attempt to keep steroid production steady, several alterations of the culture medium have been investigated, including addition of lipoproteins [19, 20], low maintenance doses of LH [19], and reduction in oxygen tension in order to minimize damage by free-radicals [4, 19, 21].

In this study an attempt was made to stabilize cytochrome *P*450 by the addition of dexamethasone and metyrapone, substances known to induce and stabilize cytochrome *P*450 in cultures of hepatocytes [5–7]. Dexamethasone is known to induce the CYP3A-group in hepatocyte cultures [22]. Furthermore, reports on dexamethasone treatment of Leydig cells demonstrated a high content of glucocorticoid receptors [23, 24] and a decrease in LH/hCG receptor content upon treatment with dexamethasone [25, 26]. Metyrapone is reported to maintain the content of cytochrome *P*450 in hepatocytes in culture by inhibiting the degradation of cytochrome *P*450 [6].

The present study showed that dexamethasone stabilized or induced the total amount of cytochrome *P*450 of Leydig cells in culture, the effect being dependent on the age of the rats as well as the time of culture. The effect was fast in pubertal and adult rats, and slower in prepubertal rats. After 44 h of culture (22 h for 10-week-old rats) the cytochrome *P*450 content was not detectable at any of the ages. Dexamethasone was reported to repress CYP11A1 *de novo* synthesis and mRNA levels in mouse Leydig cells [27], but did not affect CYP17 mRNA [3]. However, Welsh *et al.* [28] observed an inhibition of the CYP17 activity in rat Leydig cells. Thus the effect of dexamethasone on cytochrome *P*450 seems to depend on the species tested. However, the effect observed in this study, may be due to induction or stabilization of cytochrome *P*450 enzymes involved in hydroxylation of testosterone, or of cytochrome *P*450 enzymes catalyzing non-steroidogenic oxidation [29]. Metyrapone had no effect on cytochrome *P*450 content.

In a previous study [10] it was reported that in adult rat Leydig cells dexamethasone decreased 3β-HSD activity in a dose-dependent manner after 44 h of

culture. In agreement with this finding, Payne and Sha [3] observed a decrease in the 3β-HSD mRNA level when adult mouse Leydig cells were treated with dexamethasone. In the present study it was examined whether the decrease in 3β-HSD activity was an age related phenomenon. We found that dexamethasone decreased 3β-HSD activity in Leydig cells in rats 3–10 weeks old. The effect on Leydig cells from 10-week-old rats seemed to be delayed compared to younger animals, as 3β-HSD activity was not significantly inhibited until after 44 h of culture, whereas the decrease was already observed after 22 h of culture in Leydig cells from younger rats. On the other hand, the effect in Leydig cells from 3-week-old rats did not last for 44 h.

In Leydig cells from prepubertal and pubertal animals dexamethasone decreased the basal testosterone secretion after 22 h but not after 44 h of culture and had no effect on LH-stimulated testosterone production. In rats 7 weeks old, the age of transition from pubertal to adult animals, dexamethasone decreased the basal and LH-stimulated testosterone production after 22 and 44 h of culture. In adult rats dexamethasone had no effect on basal testosterone secretion, and furthermore, the effect on the LH-stimulated testosterone level did not appear until after 44 h of culture, the delay being in agreement with observations on 3β-HSD activity. However, others have reported that treatment with dexamethasone *in vivo* or *in vitro* for a prolonged time to hypophysectomized or adrenectomized prepubertal, pubertal or adult rats partially blocked the stimulatory effect of LH/hCG on cAMP and testosterone levels [23, 25, 26, 28].

The observed testosterone production in this study may be a result of both biosynthesis and degradation. The degradation of testosterone in Leydig cells from adult rats was catalyzed by 7α- and 6α-testosterone hydroxylases [2]. 7α-Hydroxylase was reported to be induced by dexamethasone in an *in vivo* study in hepatocytes [30], while induction was not observed in an *in vitro* study [7]. The 5α-reductase, responsible for the conversion of testosterone to dihydrotestosterone in young rats, was not inducible by dexamethasone in cultures of hepatocytes [31]. It seems that the effect of dexamethasone on the LH-stimulated testosterone production in Leydig cells was absent in cells from young rats and did not appear until after 44 h of culture in cells from adult animals. Induction of testosterone catabolism by induced *P*450 testosterone hydroxylases might be involved in the decrease of the estimated testosterone production in adult rats.

The basal and LH-stimulated testosterone synthesis were very low in Leydig cells from rats 3 weeks old. The capacity of Leydig cells to synthesize testosterone increased with age, reaching a maximum at rats 7 weeks old. Between the age of 7 and 10 weeks the testosterone level decreased by approx. 60%. These observations are in agreement with other reports on development of testosterone synthesis [32–34], except for the decrease in testosterone synthesis between the 7th and 10th week. The highest 3β-HSD activity was observed in Leydig cells of rats 5 weeks old, as shown previously [35, 36].

Dexamethasone seems to affect Leydig cells in several ways, the effects being on LH/hCG receptor content, transcription of mRNA, and stabilization or induction of the cytochrome *P*450 content.

An assembled evaluation of the effects of dexamethasone during development has never been performed. These results indicate developmental differences in the response to *in vitro* dexamethasone treatment. The effects of dexamethasone during culture of Leydig cells from young animals seem to develop more rapidly than in cultures of Leydig cells from older rats, but the effects were transient, as most of them disappeared after 22 h of treatment.

Acknowledgements—We thank Jytte Palmgren-Salomonsen for expert technical assistance and are grateful to Anette Kristensen for advice and discussions. This work was supported by the Novo Fund. Some of the results of the present paper were presented at the XIIth North American Testis Workshop, 1993.

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