Kinetic and autoradiographic studies on binding of hCG to the testicular LH receptors of neonatal rats

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Summary. The properties of hCG binding to LH receptors of the neonatal (5-day-old) rat testis were analysed and compared with those of the adult testis. The equilibrium association constants (K_a) of hCG-binding were similar at both ages, $2\text{--}4 \times 10^{10} \text{ M}^{-1}$. In contrast, kinetic binding studies revealed that the association and dissociation rate constants of hCG binding were more rapid in the neonatal testis. Likewise, it was observed that the progression from loose (easily dissociable) to tight (non-dissociable) binding was less complete in the young than in the adult testis. Autoradiography of ^{125}I -labelled hCG binding to interstitial cell suspensions at the two ages showed that the gonadotrophin binding per Leydig cell was about 50% lower in the neonatal testis. Conversely, since the surface area of adult Leydig cells was about 4-fold larger, the receptor density appeared to be higher in the neonatal Leydig cells. The rapid recovery of LH receptors after hCG stimulation, typical of the neonatal cells, was due to rapid replenishment of binding in the cells initially occupied by the injected hormone, rather than to an hCG-induced increase of Leydig cell number. Finally, in-vivo experiments with cycloheximide revealed that the rapid recovery of LH receptors was dependent on protein synthesis. These differences in the kinetics of neonatal testicular LH receptor turnover may be involved in the unique functional features of the fetal–neonatal growth phase of rat testicular Leydig cells.

Introduction

Two distinct populations of Leydig cells are present in the testis during different periods of development (see Huhtaniemi, Warren & Catt, 1984, 1985). The first, the fetal–neonatal growth phase, appears in the rat testis during the last third of gestation and disappears gradually over the first 2 weeks of postnatal life. The second, the adult growth phase, appears during puberty and persists throughout adult life. Although the main function of both Leydig cell populations is the same, i.e. to produce androgens, we have identified distinct functional differences in their hormonal regulation and steroidogenesis. These differences include a higher testosterone production capacity (Tapanainen, Kuopio, Pelliniemi & Huhtaniemi, 1984) and apparent lack of LH receptor down-regulation (Huhtaniemi, Nozu, Warren, Dufau & Catt, 1982) and steroidogenic desensitization (Huhtaniemi, Warren, Apter & Catt, 1983) in the fetal–neonatal Leydig cells after gonadotrophic stimulation. We have extended our earlier findings in the present study on the special characteristics of LH receptor binding in the Leydig cells of neonatal rats by using kinetic receptor measurements and autoradiography.

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Materials and Methods

Hormone preparations. Highly purified hCG (CR-121) with a biological activity of 13 500 i.u./mg by bioassay was prepared by Dr R. Canfield (Department of Medicine, Columbia University, NY, U.S.A.). Partly purified hCG (Pregnyl; 3000 i.u./mg by bioassay) was purchased from Organon (Oss, The Netherlands).

Animals and treatments. Male rats of the Sprague-Dawley strain were used at the age of 4–5 days or 70–90 days. The neonatal rats were kept in groups of 10 with each lactating mother, and the adults as 10 per cage. Lighting schedule (14 h on, 10 h off) and temperature (23°C) were controlled, and water and laboratory animal chow were available *ad libitum*.

Testes of 5-day-old and 70-day-old rats were used for the kinetic hCG binding measurements.

In one experiment, 50 4-day-old male rats were injected s.c. with 600 i.u. hCG/kg (Pregnyl) and killed 3 days later. Saline-injected animals (50) of the same age served as controls. Interstitial cell suspensions were prepared of testes of these animals, as well as of testes of 5 adult control animals (Dufau & Catt, 1975). The cell suspensions were allowed to bind radioiodinated hCG *in vitro* (see below), and thereafter processed for light microscopic autoradiography (see below).

In another experiment, groups of 4-day-old rats were injected as above with hCG, and killed 72 h later. Half of these animals were, in addition, injected with 10 mg cycloheximide/kg (Sigma Chemical Co., St Louis, MO, U.S.A.) at 48 and 60 h in relation to the hCG injection. The rest of the animals were injected twice with the vehicle, 25 μ l 50% ethanol. The purpose of this procedure was to inhibit protein synthesis between 48 and 72 h after hCG, which is known to represent the fastest phase of receptor recovery after hCG (Huhtaniemi, Katikineni & Catt, 1981).

Preparation and characterization of radioiodinated hCG. The highly purified preparation of hCG (CR-121) was radioiodinated by the lactoperoxidase-solid phase method as described by Karonen, Mörsky, Siren & Seuderling (1975). Separation of the hormone from free iodine was carried out using a 0.6 $\times$ 10 cm column of BioGel P-60 (BioRad, Richmond, CA, U.S.A.). The specific activity of each batch of labelled hormone was determined by self-displacement analysis in the homologous radioligand-receptor assay, using rat testis homogenate (Catt, Ketelslegers & Dufau, 1976). A correction was also made for the fraction of radioactivity associated with biologically active hormone, as measured in each labelled preparation by determining the maximum specific binding of a sample of the tracer to an excess of rat testis receptors (Catt *et al.*, 1976). The biologically active fraction varied between 50 and 60%, and the corrected specific activities were 60–80 Ci/g.

Equilibrium binding of hCG to testicular LH receptors. Rat testes were homogenized in Dulbecco's phosphate-buffered saline (pH 7.4), containing 0.1% bovine serum albumin (PBS-BSA), 10 ml/g tissue. The homogenate was centrifuged at 600 g for 10 min, and the supernatant was further centrifuged at 25 000 g for 20 min. The pellet was resuspended in 10 ml PBS-BSA/g original tissue, and is referred to as testicular homogenate. Aliquants of testicular homogenate (100 μ l) were incubated with the iodinated preparation of hCG for 16 h at room temperature in a final volume of 0.25 ml. Nonspecific binding was determined in matched samples in the presence of a 1000-fold molar excess of hCG (Pregnyl). Bound and unbound hormone were separated by 15-fold dilution of the samples with ice-cold PBS-BSA and centrifugation at 1500 g for 20 min, and the bound radioactivity was measured in a γ -spectrometer.

Kinetic studies on hCG binding. The association rate constants of binding (k_1) were determined as described previously (Huhtaniemi & Catt, 1981), by incubating 100- μ l samples of the testis homogenate with a high concentration (about 200 pmol/l) of iodinated hCG at 37°C, in a final volume of 0.25 ml. Incubations were stopped at selected intervals up to 240 min by diluting the samples 15-fold with ice-cold PBS-BSA, and immediately centrifuged at 4°C for 20 min (1500 g). Nonspecific binding of matched samples was determined as above.

The dissociation rate constants and half-times of binding of the iodinated hormone preparations were measured by first incubating 100- μ l samples of testis homogenate at 24°C for 16 h with about 200 pmol iodinated hCG/l. Samples having a 1000-fold excess of unlabelled hCG were used to monitor the nonspecific binding. Unbound hormone was removed by dilution and centrifugation, followed by two further washes with 15 volumes of PBS-BSA. The pellets were then reconstituted to the original volume (0.25 ml) of PBS-BSA containing 2000 i.u. hCG/ml (Pregnyl) and incubated at 37°C for up to 24 h. At selected intervals, samples were removed, the reaction was stopped by dilution and centrifugation (as above), and the radioactivity remaining in the pellet was measured.

Assessment of the loose component of hCG binding. Testis homogenates were preincubated with 125 I-labelled hCG at 37°C for 15–240 min. Further binding of the iodinated hormone was then stopped in one set of samples by the addition of a 1000-fold excess of unlabelled hCG. The samples were thoroughly mixed by vortexing and further incubated at 37°C for 1 h, then diluted and centrifuged as described above. The other set of samples was immediately diluted and centrifuged after the first incubation. Specific binding of 125 I-labelled hCG remaining in the pellets was measured as described above.

Analysis of binding data. An interactive computer program (MLAB) with differential equation solving abilities (Rodbard & Hutt, 1974; Ketelslegers, Knott & Catt, 1975) was used to perform all curve fittings and calculations. This program, using a PDP-10 time sharing computer, also provided the original graphic output of the illustrations presented in this report. In the calculations, a molecular weight of 37 000 was used for hCG (Canfield, Birken, Morse & Morgan, 1976). Statistical analyses were performed using Student's *t* tests for unpaired samples.

Binding of iodinated hCG to interstitial cell suspensions prepared from neonatal and adult testes. This study was carried out to assess the number of LH receptors per Leydig cell in the neonatal rat

Fig. 1. Association of 125 I-labelled hCG at 37°C with LH receptors present in homogenates of neonatal and adult rat testes. The amount of homogenate represents 10 mg of the original tissue at each point, and the concentration of iodinated hCG was 200 pmol/l. k_1 = association rate constant. Each point is the mean $\pm$ s.e.m. ($n = 3$). One of two similar experiments is presented.

testes before and after hCG stimulation. The animals (see above) were killed 3 days after hCG injection by decapitation, and decapsulated testes were dispersed with collagenase to prepare crude interstitial cell suspensions (Dufau & Catt, 1975). Interstitial cells prepared from 5 adult testes served as controls.

The 3 cell suspensions were stained for 3β -hydroxysteroid dehydrogenase (Wiebe, 1976), and appropriate dilutions were made to yield about 10^6 positive cells per ml. The cells were incubated in Medium 199 + 0.1% BSA in the presence of 5×10^5 c.p.m. of ^{125}I -labelled hCG for 2 h at 37°C in the presence or absence of 2000 i.u. unlabelled hCG/ml. After incubation, the cells were washed 4 times with 5 ml ice-cold Medium 199, resuspended in 100 μl of the medium, and smeared on microscopic slides. After drying in air, the cells on slides were fixed in 10% formaldehyde in PBS for 20 min, and processed for autoradiography (see below).

Autoradiography. The microscopic slides containing the fixed cells were washed for 5 min in PBS, and coated with Kodak NTB-3 photomicrographic emulsion (Eastman Kodak Co., Rochester, NY, U.S.A.) as described in detail earlier (Rajaniemi & Midgley, 1975). After exposure for 3 weeks at -18°C , the emulsion was developed, and fixed, and the cells were stained and haematoxylin and eosin (Rajaniemi & Midgley, 1975). The slides were photographed at $\times 450$ magnification, and the grains were counted on the photographs. One hundred grain-positive cells were counted in each group of samples, and the percentage distribution of the number of grains per cell (to the nearest 10) was calculated.

Testosterone assay. Testosterone of the testis homogenates was measured by radioimmunoassay as described by Hammond, Viinikka & Vihko (1977). The sensitivity of the assay was about 5 pg/assay tube, and the intra- and interassay CVs were 5% and 11%, respectively.

Fig. 2. Dissociation of specifically bound ^{125}I -labelled hCG from homogenates of neonatal and adult testes. The association took place at 24°C for 16 h, whereafter the unbound hormone was removed by washing and centrifugation. The dissociation from reconstituted pellets was measured in the presence of a 1000-fold excess of unlabelled hCG at 37°C . $T_{1/2}$ = half-time of binding. Each point is the mean $\pm$ s.e.m. of triplicate determinations. The results presented in the figure are from one of two similar experiments.

Results

Association and dissociation kinetics of binding of ^{125}I -labelled hCG

As previously (Huhtaniemi *et al.*, 1981), the K_a values of hCG binding to homogenates of neonatal and adult rat testes were similar, $2\text{--}4 \times 10^{10} \text{ M}^{-1}$ (results not shown). The kinetics of the hormone binding to the tissue homogenates were further analysed in the present experiments. In two similar incubations, the association rate constants (k_1) were 1.6 and $2.3 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ for the neonatal testes, and 1.0 and $1.1 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ for the adult testes (Fig. 1). Dissociation curves of hCG binding to the two tissue samples are presented in Fig. 2. Two slopes of dissociation of bound hCG were seen at both ages, and the dissociation rate constants for the fast and slow slopes were $0.61\text{--}0.77 \times 10^{-3}$ and $0.21\text{--}0.26 \times 10^{-3} \text{ min}^{-1}$ for the neonatal testes, and $0.55\text{--}0.77 \times 10^{-3}$ and $0.096\text{--}0.14 \times 10^{-3} \text{ min}^{-1}$ for the adult testes in the two similar experiments performed. The half-times of dissociation were $8.5\text{--}10 \text{ h}$ for the neonatal testes and $20\text{--}34 \text{ h}$ for the adult testes.

Progression of hCG-receptor complexes from loose to tight binding

In one set of kinetic experiments, the association of hCG with receptors was stopped by dilution of the sample with ice-cold buffer and immediate centrifugation, or by addition of excess of non-radioactive hormone, thorough mixing and continuation of the incubation for another hour (followed then by dilution and centrifugation). Different binding data were observed: the specific binding was higher with addition of excess unlabelled hCG in the adult testis homogenates during the first 30 min of the hCG-receptor association, but in neonatal samples this phenomenon persisted throughout the 240-min incubation time. As before (Katikineni, Davies, Huhtaniemi & Catt, 1980), this can be explained by dissociation of some of the loosely bound hormone in the dilution method, and progression of these hormone-receptor complexes from loose to tight binding if the incubation is allowed to proceed for another hour in the presence of excess non-radioactive hormone. Hence, the loosely associated fraction of bound hCG is higher and more persistent in the neonatal testis homogenates (Fig. 3).

Degradation of hCG in neonatal and adult testis homogenates

Since the differences in binding kinetics observed in the previous experiments could be explained by differential degradation of the tracer hormones by tissue homogenates, this was controlled in a series of incubations. About 20 mg homogenized testis tissue from both ages were incubated with $5 \times 10^5 \text{ c.p.m.}$ of iodinated hCG in a total volume of 300 μl for 3 h at 37°C . Appropriate controls were incubated in the presence of excess unlabelled hormone (50 i.u. hCG (Pregnyl)). After incubation, the unbound hormone was separated by centrifugation, and tested with another set of adult testis homogenates, to determine the percentage of biologically active hormone remaining free in the first incubation. After incubation at room temperature overnight, the specific binding was $38.9 \pm 1.4\%$ (preincubation with neonatal tissue) and $39.0 \pm 0.84\%$ (adult tissue) (mean $\pm$ s.e.m., $n = 3$) of the binding measured with freshly labelled (non-preincubated) hormone. This indicates that no differences are seen in the amount of degradation of hCG by testis homogenates of the two ages.

Autoradiography of iodinated hCG binding to interstitial cell suspensions

Figure 4 shows an autoradiographic picture of iodinated hCG binding to interstitial cell suspensions of neonatal and adult testes. The Leydig cell content of the suspensions, as measured by 3β -hydroxysteroid dehydrogenase staining, was $20\text{--}30\%$. Therefore, both grain-positive (tens of

Fig. 3. Effect of association time and the method of interruption of association on the amount of specific binding of ^{125}I -labelled hCG to testis homogenates of neonatal ($\circ$ — $\circ$) and adult ($\bullet$ — $\bullet$) rats. The testis homogenates were incubated at 37°C in the presence of 200 pmol iodinated hCG/l, in the presence or absence of 50 i.u. hCG (Pregnyl) (to assess the nonspecific binding). After selected intervals, the association was stopped by dilution and centrifugation (see 'Methods') or by adding 50 i.u. hCG (Pregnyl) and continuing the incubation for another 60 min (followed by dilution and centrifugation). Binding measured by the former method was taken as 100%, and the binding measured by the second method is expressed as % thereof. Asterisks indicate points where the amounts of binding measured by the 2 methods were significantly ($P < 0.05$) different. Values are mean $\pm$ s.e.m. of triplicate measurements for data of one of two similar experiments.

grains per cell) and negative (a few grains per cell, and no difference from background) nucleated cells are seen. Only the latter type of cells were identified when cells were labelled in the presence of excess non-radioactive hCG (results not shown). The distribution of grain counts per positive cell in the 3 different samples analysed is shown in Fig. 5. The number of grains was 92.5 ± 3.6 (mean $\pm$ s.e.m., $n = 100$) in the adult testis, 40.9 ± 1.1 in cells from control neonatal testes, and 41.0 ± 1.2 in the neonatal cells prepared 3 days after hCG injection.

We also estimated the effect of hCG injection on cell size in the grain-positive cells. This was done by selecting grain-positive cells at random, and multiplying their longest and shortest diameters. The figure, a rough index of Leydig cell surface area, was 105 ± 8.5 (mean $\pm$ s.e.m., $n = 50$) for the adult cells, 23.5 ± 1.2 for the control neonatal cells, and 29.1 ± 1.4 for the cells after hCG. The surface area of Leydig cells of adults is therefore ~ 4 times larger than that of cells from neonatal rats and the hCG treatment increases the surface area of the neonatal cells by 24%. Finally, when these figures and the grain counts were combined, the number of LH receptors per surface area appeared to be about double in the neonatal Leydig cells compared with those of the adult animals.

Effect of cycloheximide on recovery of LH receptors after hCG injection

The nature of the rapid LH receptor recovery between 2 and 3 days after hCG injection

Fig. 4. Light-microscopic autoradiographic pictures of binding of ^{125}I -labelled hCG to interstitial cell suspensions prepared from (a) neonatal and (b) adult rats. $\times 450$.

Fig. 5. Grain distribution in autoradiography of binding of ^{125}I -labelled hCG to interstitial cell suspensions prepared from control adult and neonatal (7 days) testes, and from neonatal testes 3 days after the injection of 600 i.u. hCG/kg body weight. The numbers of grains per cell were 92.5 ± 3.6 ($\pm$ s.e.) in the adult testes, 40.9 ± 1.1 in the control neonatal testes, and 41.0 ± 1.2 in the neonatal testes after hCG injection.

(Huhtaniemi *et al.*, 1981) was studied by inhibiting in-vivo protein synthesis with cycloheximide. LH receptors were measured in the testes of animals injected with hCG alone and with hCG plus cycloheximide on Day 3 of the experiment, at the time when full receptor recovery is reached at this age. Testicular hCG binding in the hCG-injected animals was 0.45 ± 0.027 fmol/testis (mean $\pm$ s.e.m., $n = 20$), but only 0.26 ± 0.041 fmol/testis ($n = 12$) in the cycloheximide-treated animals ($P < 0.01$). By contrast, no difference was observed in the testicular testosterone content, which was 3.09 ± 0.21 ng/testis in the hCG-treated animals and 3.21 ± 0.46 ng/g in the animals also receiving cycloheximide. This experiment was considered to provide evidence for dependence on protein synthesis of the rapid testicular LH receptor recovery that is typical of the neonatal animals.

Discussion

Despite similar equilibrium binding, we observed that the association and dissociation of hCG with testicular receptors was faster in the young than in adult animals. Besides the receptor molecule, the difference may reside in the membrane microenvironment which is known to influence affinity of the hormone–receptor interaction (Huhtaniemi & Catt, 1981). The third observation of the kinetic binding measurements, i.e. the higher proportion and persistence of ‘loose’ hCG binding in the neonatal testis, is of particular interest. The gradual progression from loose to more tight binding has been suggested to precede the process of receptor-mediated hormone internalization (van der Gugten, Waters, Murthy & Friesen, 1980; Katikineni *et al.*, 1980). Therefore, gonadotrophin–receptor internalization may not be as extensive in the Leydig cells of neonates as in adults. In fact, the rapid replenishment of LH binding after hCG-induced receptor occupancy (Huhtaniemi *et al.*, 1981), and the lack of heterologous loss of prolactin receptors upon gonadotrophin stimulation (Huhtaniemi *et al.*, 1982), suggest that the elimination of gonadotrophin–receptor complexes in the Leydig cells of fetuses may proceed by different mechanisms. Shedding of the hormone ligand and/or hormone–receptor complexes from the plasma membrane is a possible alternative to internalization.

The amount of hCG binding per unit weight of neonatal testis is clearly lower than that of the adult testes (Huhtaniemi *et al.*, 1981; Clausen, Purvis & Hansson, 1981). These data have been difficult to interpret because of differences in Leydig cell densities at the different ages (Huhtaniemi *et al.*, 1981; Tapanainen *et al.*, 1984). Leydig cell suspensions prepared from neonatal and adult testes have similar binding of hCG per β -hydroxysteroid dehydrogenase-positive cell (Huhtaniemi *et al.*, 1981), but these measurements are compromised by the known developmental differences in the activity of this steroidogenic enzyme (Wiebe, 1976; Paz, Winter, Reyes & Faiman, 1980; Clausen *et al.*, 1981). Data on grain counts showing distribution of ^{125}I -labelled hCG in interstitial cells may give more reliable information about Leydig cell LH receptor numbers at the two ages studied. The amount of grains per Leydig cell in the neonatal testis was about one-half of that of the adult, as also shown by Clausen *et al.* (1981). In contrast, the diameter of the adult cells was about double, and the surface area about 4-fold in comparison with the young cells. Consequently, the density of LH receptors appeared to be about 2-fold higher in the neonatal than in the adult Leydig cells. Also the steroidogenic capacity of the fetal–neonatal Leydig cells is higher than in the adult cells (Tapanainen *et al.*, 1984), despite their smaller size. Whether the LH receptor density is related to the steroidogenic activity of the fetal population of Leydig cells remains to be resolved.

The LH receptor recovery after gonadotrophin stimulation is rapid in the neonatal rat testis; complete recovery of binding occurs in 3 days compared with 14 days in the adult (Huhtaniemi *et al.*, 1982). It has not been clear whether the fast recovery is due to faster appearance of new receptors in the pre-existing Leydig cells or to induction by hCG of a new population of Leydig cells and new receptors; the Leydig cell mass increases in 3 days almost 2-fold after hCG stimulation of the 5-day-old rat (Huhtaniemi *et al.*, 1981). In the latter case, if the original Leydig cells respond to

hCG with the true adult-type LH receptor down-regulation and slow rate of recovery, all the replenished binding must reside in the newly differentiated Leydig cells. In this case, autoradiography should demonstrate highly labelled cells, to compensate for the fact that most of the cells have lost their LH receptors as a result of down-regulation. However, this was not the case, since the number of grains per cell was the same before and after the hCG injection. It can only mean that both the 'old' and the 'new' cells have the same number of LH receptors and that the recovery of receptors after hCG must be fast in all Leydig cells of the neonatal testis. Since cycloheximide delayed the reappearance of binding, this phenomenon must be dependent on protein synthesis, i.e. synthesis of new binding sites. It also indicates that, as in adults (Katikineni *et al.*, 1980), hCG binding is practically irreversible; after binding and elimination of the hormone, synthesis of new receptor molecules is needed for replenishment of binding.

In conclusion, we have demonstrated that the fetal–neonatal population of Leydig cells displays very rapid turnover of LH receptors upon gonadotrophic stimulation. This phenomenon is accompanied by faster association and dissociation kinetics of hCG binding and persistence of the loosely bound component of hormone–receptor complexes. Taken together, the observations suggest that the mechanisms of LH receptor turnover could be different at these two ages and more detailed studies are required to elucidate further the mechanisms involved in hormone action mediated by plasma membrane receptors.

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