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Regulation of 3β-Hydroxysteroid Dehydrogenase in Gonadotropin-Induced Steroidogenic Desensitization of Leydig Cells

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ABSTRACT

3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerases (3β-HSD) are enzymes that catalyze the conversion of Δ^5 to Δ^4 steroids in the gonads and adrenal for the biosynthesis of sex steroid and corticoids. In gonadotropin-desensitized Leydig cells, from rats treated with high doses of human CG (hCG), testosterone production is markedly reduced, a finding that was attributed in part to reduction of CYP17 expression. In this study, we present evidence for an additional steroidogenic lesion induced by gonadotropin. Using differential display analysis of messenger RNA (mRNA) from Leydig cells of rats treated with a single desensitizing dose of hCG (2.5 μg), we found that transcripts for type I and type II 3β-HSD were substantially (5- to 8-fold) down-regulated. This major reduction, confirmed by RNase protection assay, was observed at the high hCG dose (2.5 μg), whereas minor or no change was found at lower doses (0.01 and 0.1 μg). In contrast, 3β-HSD mRNA transcripts were not changed in luteinized ovaries of pseudopregnant rats treated with 2.5 μg hCG. The down-regulation of 3β-HSD mRNA in the Leydig cell resulted from changes at the transcriptional level. Western blot analysis showed 3β-HSD protein was significantly reduced by hCG treatment, with changes that were coincidental with the reduction of enzyme activity and temporally consistent with the reduction of 3β-HSD mRNA but independent of LH receptor down-regulation. The reduction of 3β-HSD mRNA resulting from transcriptional inhibition of gene expression, and the consequent reduction of 3β-HSD activity could contribute to the inhibition of androgen production in gonadotropin-induced steroidogenic desensitization of Leydig cells. The gender-specific regulation of 3β-HSD by hCG reflects differential transcriptional regulation of the enzymes to accommodate physiological hormonal requirements and reproductive function. (*Endocrinology* **139:** 4496–4505, 1998)

THE ENZYME 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), which converts 3β-OH-5 ene to 3β-OH-4 ene steroids, plays a pivotal role in the biosynthesis of steroid hormones during gonadal development and differentiation process (1–3). In the ovary, 3β-HSD catalyzes the final step in progesterone biosynthesis. In the testis, 3β-HSD is required for testosterone production. Various 3β-HSD isoforms have been cloned from the gonads, liver, and other peripheral tissues in the human, rat, and mouse (2, 4, 5). In the rat, there are four types of 3β-HSD; only types I and II are expressed in gonads (1, 4). They both encode a 372-amino acid protein and share 93.8% sequence homology. *In vitro* studies have demonstrated that the activity of the type I enzyme is higher than that of the type II enzyme, a finding attributed to the absence of a putative membrane spanning domain in the type II sequence, which leads to the lower affinity to substrates (6).

Steroidogenesis in the Leydig cell depends on the action of gonadotropin [LH/human CG (hCG)] exerted through its homologous receptor, to induce coupling functions and activation predominantly of the adenylate cyclase/protein kinase pathway (7–9). In the adult testis, gonadotropin induces a dual control of Leydig cell function. Low doses of LH/hCG maintain LH/hCG receptor and steroidogenic enzymes, whereas higher doses of the hormones cause receptor down-regulation and desensitization of the steroid biosynthetic pathway with marked reduction of testosterone production. Previous studies have demonstrated a blockade at the site of conversion of progesterone to androgens; this reduction was partly attributable to decrease of CYP17 (17α-hydroxylase/17, 20 lyase) expression (8, 10). The fate of other relevant enzymes in the steroid pathway including 3β-HSD in this desensitizing process remained to be elucidated. To investigate regulation of 3β-HSD by desensitizing doses of gonadotropin in Leydig cell, *in vivo* and *in vitro* approaches were used to mimic physiological and pharmacological conditions. Using differential display analysis of Leydig cell messenger RNAs (mRNAs), we have demonstrated rapid hCG-induced negative regulation of type I and II mRNA of 3β-HSD in the Leydig cells of adult rats that is independent of the down-regulation of the LH receptors.

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Materials and Methods

Animal treatment and Leydig cell preparation

Adult male rats (200–250 g) (Charles River Laboratories, Inc., Wilmington, MA) were housed in pathogen-free, temperature- and light-controlled conditions (20 C; alternating 14-h light, 10-h dark cycles). All animal studies were approved by the NICHD Animal and Care and Use Committee (Protocols 97–039 and 94–041). Gonadotropin-induced LH receptor (LHR) down-regulation and steroidogenic desensitization of Leydig cells was produced as previously described (10) by administration of a single sc injection of hCG (Pregnyl, Organon, West Orange, NJ) 25 IU (equivalent to 2.5 μg of purified hCG) in 100 μl of PBS, pH 7.4. hCG IU are equivalent to hCG USP (United States Pharmacopea Unit). Control animals were injected with vehicle alone. Animals were

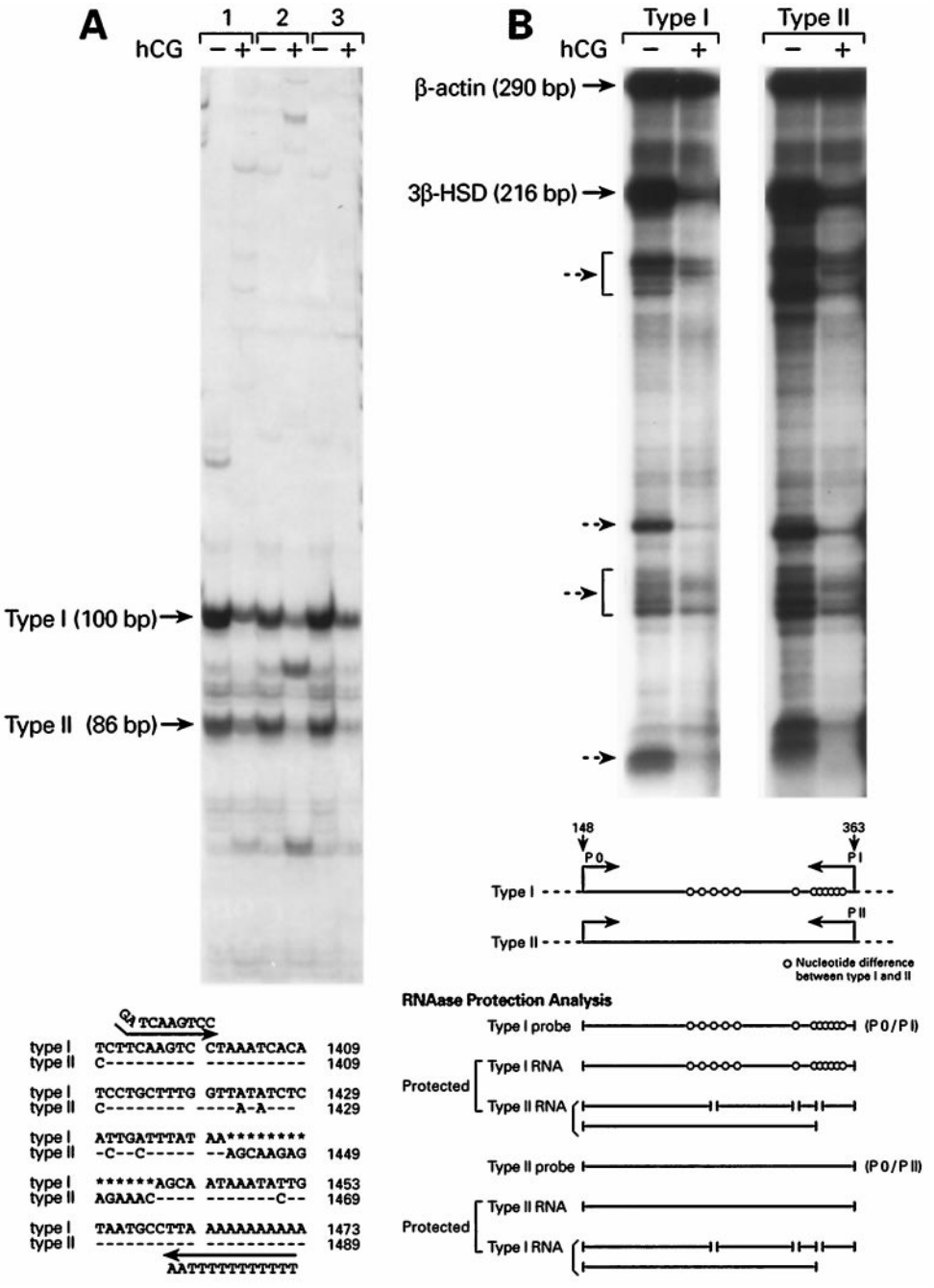

FIG. 1. Down-regulation of 3β-HSD by hCG identified by mRNA differential display analysis (A) and confirmed by RNase protection assay (B) in rat Leydig cells. A, Leydig cells were obtained from rats treated with 2.5 μg of hCG and untreated controls 24 h after injection of a single sc dose of hCG or vehicle (control). Using differential display analysis, three sets (with and without hCG treatment) of total RNA samples (1–3) were reverse transcribed with the oligo 5-dT11AA-3 primer, followed by PCR with ^{33}P-dATP and primers of 5-dT11AA-3 and 5-GATCAAGTCC-3 (*lower panel*). The two bands indicated by *arrows* (100 bp and 86 bp) correspond to 3β-HSD type I and II mRNA, respectively; these were confirmed by sequence (*lower panel*). The *dashes* (—) and *asterisks* (*) respectively indicate the identical and missing nucleotides. B, Total RNA samples (10 μg) were hybridized to ^{32}P-labeled type I or type II of 3β-HSD, simultaneously with β-actin probes (internal control). Protected fragments were resolved on 6% denatured sequencing gels. The cRNA probes for 3β-HSD complementary to the coding region from 148 nt to 363 nt relative to the translational start site (*lower panel*) were derived from RT-PCR with sets of primers: P0/PI for type I and P0/PII for type II, followed by subcloning as described in the *Materials and Methods*. The top bands (290 bp) are the fragments protected by β-actin. With either 3β-HSD probe, the full-length protected fragments (216 bp) correspond to the homologous RNA species protected by the probe. The shorter protected fragments indicated by *arrows* correspond to the type II 3β-HSD mRNA in the assay with the type I probe, and the type I 3β-HSD mRNA in the assay with the type II probe. ○, Nucleotide difference between type I and type II. The representative data shown are from one of three individual experiments, each performed in triplicate.

killed by asphyxiation with CO_2 and decapitated 24 h after hCG treatment unless otherwise indicated. Leydig cells were prepared by collagenase dispersion and purified by centrifugal elutriation, as previously described (11). These cells were immediately frozen on dry ice and maintained at −70 C until extraction of RNA. *In vivo* ovarian luteinization and down-regulation of LHR and LHR mRNA was induced by sequential PMSG/hCG treatment following established protocols (12). Prepubertal 21 day old female rats (Sprague-Dawley, Charles River Laboratories, Inc.) received an sc injection with 50 IU of PMSG (a preparation rich in FSH activity) followed 65 h later by an injection of 25 IU hCG (superovulation). Six days later, the animals received a second injection of 25 IU of hCG (LHR down-regulation, steroidogenic desensitization). Twenty-four hours after the second hCG injection, animals were killed and ovaries were removed, rapidly frozen, and preserved at −70 C until RNA extraction.

Differential display analysis

Total RNA was isolated by using TRIzol RNA reagent (Gibco BRL, Gaithersburg, MD) followed by DNase I (Gibco BRL) treatment to remove DNA contamination. Samples were then reextracted by TRIzol RNA reagent and analyzed by differential display technology using Differential Display kit (Display Systems Biotechnology, Inc., Los Angeles, CA) protocol (13). Briefly, three sets of total RNA samples (300 ng for each), from animals treated with and without hCG and independently prepared three different times, were reverse transcribed with different down-stream (dT_{11} MN) primers, followed by PCR with the same downstream primer and different upstream primers (random 10-mers). PCR products with ^{33}P-dATP (2000 Ci/mmol, DuPont NEN, Boston, MA) were separated in a nondenatured 6% sequence gel. Gels were dried and evaluated by autoradiographies. Regions containing

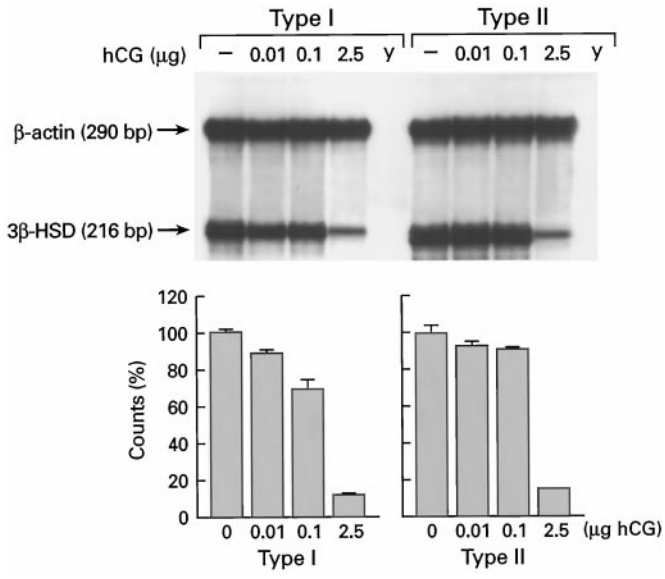

FIG. 2. Dose dependency of 3β-HSD mRNA regulated by hCG analyzed by RNase protection assay. RNA samples were prepared from Leydig cells of rats 24 h after treatment with a single sc injection of different doses (0.01, 0.1, and 2.5 μg) of hCG or vehicle (control). RNA samples (10 μg) were hybridized to the ^{32}P-labeled type I or type II 3β-HSD probe, and β-actin probe (internal control). The protected fragments were resolved on a denatured sequencing gel, recorded by autoradiography for visual display (*upper panel*) and quantified by phosphorimager (*lower panel*). Data are presented as percent of means ± SE relative to saline control of triplicates and are from one of three individual experiments. Counts of 3β-HSD were normalized by β-actin. Y = yeast transfer RNA (tRNA).

differential bands on the gel were excised. PCR products were eluted by a boiling for 5 min and incubated at 60 C for 2 h in 200 μl of 10 mM Tris-HCL/1 mM EDTA buffer. Eluted products were reamplified and purified by a single-strand conformation polymorphism gel (14). The differential bands observed in the autoradiography were eluted again, reamplified by PCR, separated on an 1.5% agarose gel, and eluted by electrophoresis. These purified differential fragments were cloned by TA-cloning kit (Invitrogen, Carlsbad, CA), and sequenced. The nucleotide sequences were analyzed and compared with sequences in the GenBank/EMBL databases using FASTA program.

RNase protection assay for 3β-HSD

The cRNA probe for 3β-HSD was generated by PCR followed by subcloning. The synthesized oligonucleotides: P0 (5-CTGCAGACAA AGGCCAAGGTG-3) and P1 (5-GATGAAGGCTGGCACACTGGCTTC-GACGCA-3) for 3β-HSD type I, P0 and P2 (5-GATGAAGGCTGGCA-CACTAGCGTGGATACC-3) for 3β-HSD type II were used to amplify the fragments corresponding to coding regions from 148 to 363 nucleotides (nt) relative to translational start site (15). The upstream primer P0 is common for both isoforms (type I and type II). The downstream primer is specific for type I (PI) or type II (PII) isoform. The sizes of the PCR products are identical but differ in their sequence. The nucleotide differences between type I and type II sequences of PCR fragments 148–363 are indicated in diagram form in Fig. 1, *right*, where the protected fragment of one isoform and unprotected fragments of the other isoform and *vice versa* are indicated also. Primers 5′-GCTACAGCT-TCACCACCACA-3′ and 5′-GGTCTTTACGGATGTCAACG-3′ located at exon 4 and 5, respectively (16), were used to amplify the rat β-actin fragment used as internal control for the RNase protection assay. The PCR products were resolved on 1.5% agarose gels and eluted by electrophoresis. The fragments were cloned into pGEM-T vector (Promega Corp., Madison, WI) and verified by sequencing. The constructed vectors were linearized by *Spe*I digestion. The cRNA probes were prepared by *in vitro* transcription with RNA polymerase T7 (Gibco BRL), labeled by α^{32}P-UTP (800 Ci/mmol, ICN Biochemicals, Inc., Costa Mesa, CA) following the protocol of the manufacturer. The procedure used for the RNase protection assay was as previously described (17). 10 μg of total RNA samples were applied for the hybridization. Ribonuclease T1 (Gibco BRL) and ribonuclease A (Amersham Life Science, Cleveland, OH) were used for the digestion of unhybridized RNA and complementary RNA (cRNA) probe. The protected fragments were resolved on a 6% sequencing gel, dried and evaluated by autoradiography. Bands were quantified by Phosphorimage Scanner (Molecular Dynamics, Inc., Sunnyvale, CA).

Actinomycin D treatment and Northern blotting analysis

Leydig cells from control and hCG treated animals were incubated with 10 μg/ml of actinomycin D for 0, 1, 3, 6, and 10 h in medium 199 containing 0.1% BSA. mRNA from Leydig cells were isolated with Fast-Track 2.0 mRNA Isolation Kit (Invitrogen). The mRNA samples (10 μg for each) were resolved in 1% agarose gels, transferred onto a nitrocellulose membrane, and hybridized to ^{32}P-labeled cRNA probe of 3β-HSD type II. Hybridization was evaluated by autoradiography and quantified by Phosphorimage Scanner. The half-lives were calculated by linear regression analysis.

Nuclear run-off assay

Nuclei from batches of 50×10^6 Leydig cells from rats injected with or without hCG were prepared as previously described (18). Briefly, the cells were washed twice with PBS, and collected by centrifugation. The cell pellets were gently vortexed while adding 2 ml lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% Nonidet P40) and incubated for 5 min on ice. The nuclei were pelleted at $500 \times g$ for 5 min and resuspended by gently vortexing in 200 storage buffer [50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA]. Nuclear RNA was labeled by *in vitro* transcription using the method of Greenberg and Bender (18) with some modification: 200 μl of nuclear extract (30×10^6 nuclei) were incubated with 20 μl [α^{32}P]-UTP (3000 Ci/mmol, 10 mCi/ml, from DuPont NEN) and 200 μl of 2 × reaction buffer containing 10 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.3 M KCl, 1 mM of each unlabeled ATP, CTP, and GTP for 30 min at 30 C. Nuclear proteins and DNA were removed by digestion with proteinase K and RNase-free DNase I. RNA was then extracted by phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated by isopropanol. RNA pellets were dissolved in 50 μl of DEPC-H_2O and pass through G-25 column (5 Prime→3 Prime Inc., Boulder, CO) to remove free nucleotides. The linearized complementary DNA (cDNA) fragments (used as templates to make cRNA probes in RNase protection assays) of 3β-HSD type I, β-actin and pGEM vector were immobilized to the nitrocellulose membranes by using a slot blot apparatus. The labeled RNA (3×10^6 cpm) were then hybridized to the membranes in 2 ml of hybridization buffer (10 mM TES, 10 mM EDTA, 0.2% SDS, and 0.3 M NaCl) for 20 h at 60 C. The membranes were washed by 2 × SSC and exposed to an x-ray film. The density of bands were quantified by phosphorimage analysis.

Western blot analysis

Protein samples (50 μg) were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were treated with blocking buffer (5% skimmed milk, 0.1% Tween-20 in PBS) for 16 h at 4 C, and then incubated with rabbit polyclonal antibody (1:2000 dilution in blocking buffer) against human placental 3β-HSD (kindly provided by Dr. Van Luu-The, MRC Group in Molecular Biology, Québec, Canada) for 2 h at room temperature. The membranes were washed three times (15 min/wash) with 0.1% Tween-20 in PBS and incubated with horseradish peroxidase conjugated goat antirabbit IgG from Gibco BRL (1:1000 dilution in blocking buffer) for 1 h at room temperature. After washing with 0.1% Tween-20 in PBS for three times (15 min/wash), the hybridization signal was detected by enhanced chemiluminescence (ECL detection system, Amersham, Arlington Heights, IL). The chemiluminescence signals which were recorded in x-ray film, were subsequently quantitated by densitometry using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA). Because of the similar molecular weights of actin and 3β- HSD (43K and 42K, respectively) it was neccesary to strip the membranes for reprobing

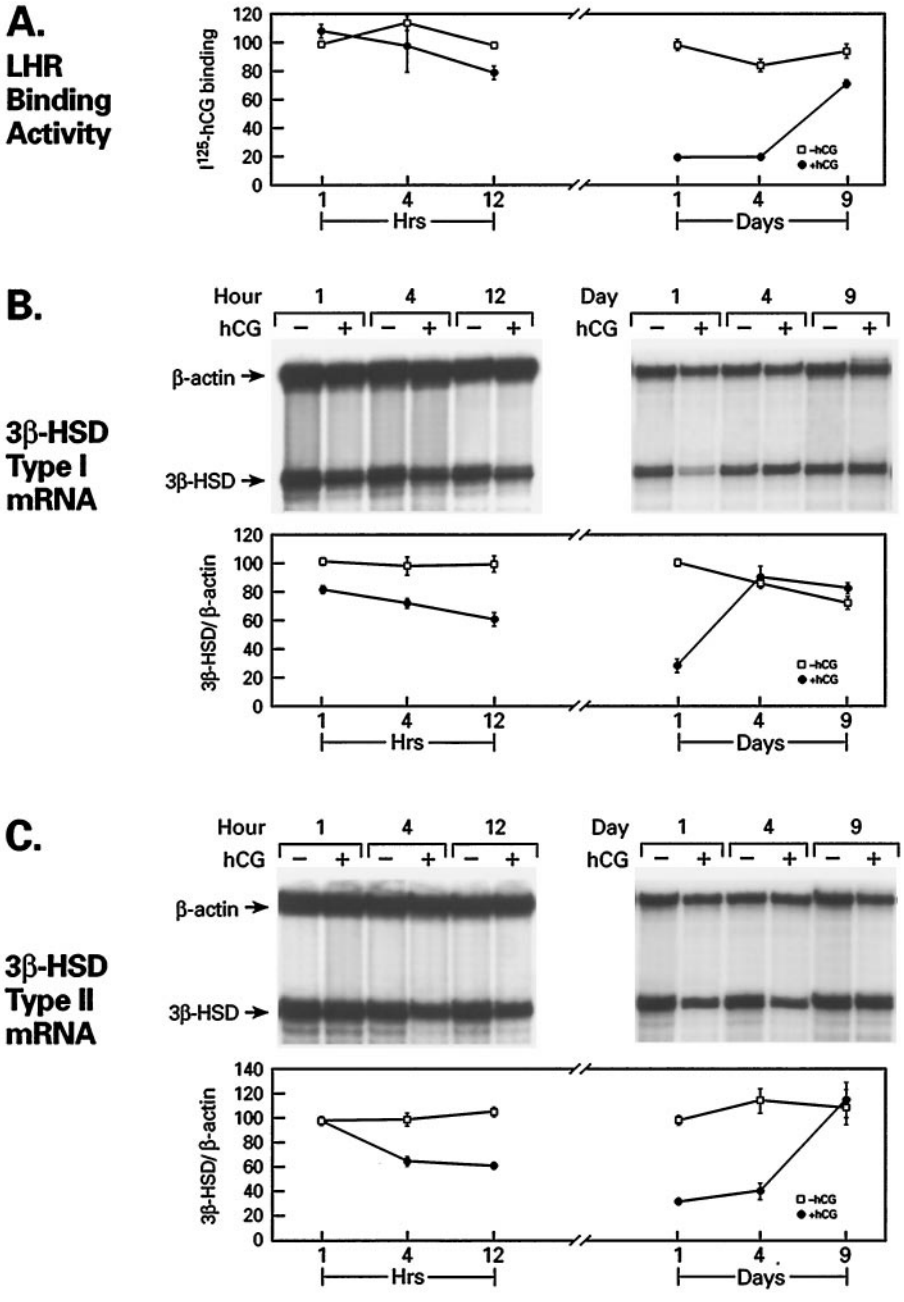

FIG. 3. Time course study of 3β-HSD mRNAs regulated by hCG analyzed by RNase protection assay and compared with LH receptor binding. Leydig cells were obtained from male rats injected with 2.5 μg of hCG after 1–12 h treatment (short term) and 1–9 days (long term). To perform a parallel study, a portion of Leydig cells was used for RNA extraction and the other for LH receptor binding assay. RNA samples (10 μg) were hybridized to the ^{32}P-labeled type I or type II 3β-HSD (B, C) together with the β-actin probe. The protected fragments were analyzed as in Fig. 2. Counts of 3β-HSD were normalized by β-actin and presented as percent of mean ± SE relative to the control. A, LH receptor binding activity was determined by binding of [^{125}I-hCG] to Leydig cells and expressed as percent of mean ± SE relative to the control. The representative data shown are from one of three individual experiments, each performed in triplicate.

with actin antiserum. The blots were stripped by incubation in buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 C for 30 min with occasional agitation. The membranes were washed with 0.1% Tween-20 in PBS two times for 15 min at 22 C and then treated with blocking buffer overnight. A mouse antiactin monoclonal antibody, which reacts with all forms of actin (Chemicon International, Inc., Temecula, CA) was employed for rehybridization (1:2000 dilution in blocking buffer), and a horseradish peroxidase conjugated goat anti-mouse IgG from Gibco BRL (1:4000 dilution in blocking buffer) was used as the second antibody (see above). Immunodetection and quantitation of the 43K actin band was performed as described above. The value of each individual 3β-HSD immunoreactive band was normalized to the corresponding actin immunoreactive band.

Enzymatic activity assay for 3β-HSD

Leydig cells isolated from hCG-treated or control rats were first incubated with 100 μg/ml of aminoglutethimide (Sigma Chemical Co., St. Louis, MO) in Medium 199 containing 0.01% BSA for 15 min to block steroid metabolism early in the pathway (cholesterol side chain cleavage) and a distally (aromatase). This was followed by incubation with addition of various concentrations (0–80 μM) of unlabeled DHEA (Sigma Chemical Co.) and a fixed concentration (1 μM) of ^{14}C-dehydroepiandrosterone [DHEA] (55.5 mCi/mmol from DuPont NEN) for 30 min. Steroids in the medium were extracted with ethyl acetate and separated on silica-coated TLC plates using chloroform and ethyl acetate (3:1, vol/vol) system as previously described (19). Plates were exposed to x-ray films (Eastman Kodak, Rochester, NY) for a visual record only. The radioactivities corresponding to unconverted steroid substrate and products in the TLC plate were detected in a phosphoscreen and quantified by Phosphorimage analysis using Phosphoimager Scanner Model Storm 860 from Molecular Dynamics, Inc. (Sunnyville, CA). Kinetic analysis was performed with ENZFITTER (Elsevier Biosoft, Amsterdam, The Netherlands). The k_{cat} was calculated from substrate concentrations, and velocity with units expressed as 0.5 $h^{-1}/10^6$ cells (20). During a

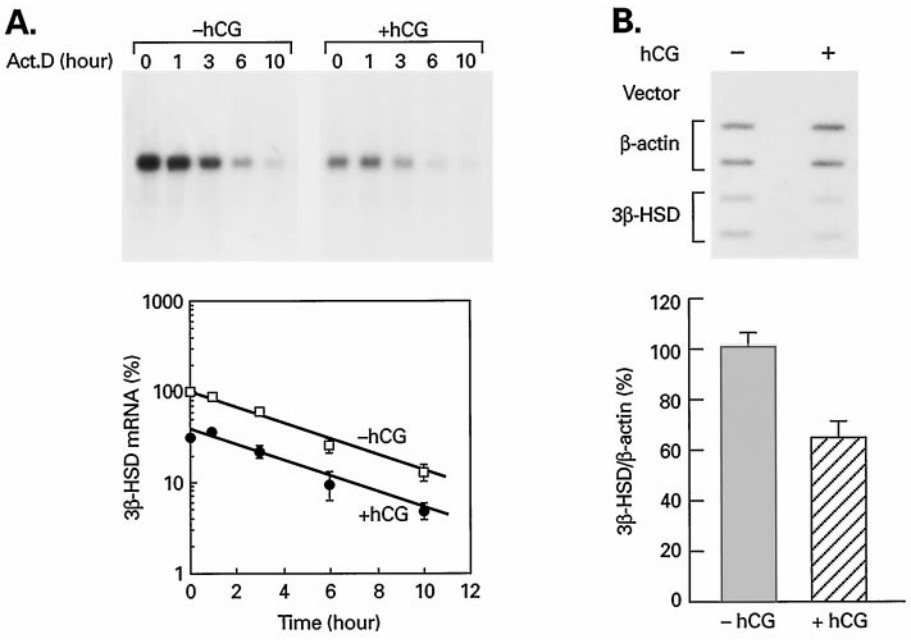

FIG. 4. Northern blot analysis of 3β-HSD mRNA from rat Leydig cells treated with Actinomycin D (A) and nuclear run-off assay (B). A, Gonadotropin-desensitized Leydig cells were obtained 12 h after treatment from rats with a single sc dose of 2.5 μg of hCG. Cells were incubated with 10 μg/ml of actinomycin D for 0, 1, 3, 6, and 10 h in medium 199 with 0.1% BSA. The mRNA samples (10 μg) were resolved on an 1% agarose gel, transferred onto a nitrocellulose membrane, and hybridized with the ^{32}P-labeled 3β-HSD cRNA probe. Results were recorded by autoradiography for visual display (*upper*) and quantified directly from the membranes by phosphorimage (*lower*). B, Nuclei were prepared from control and hCG-desensitized Leydig cells, followed by transcription assay in presence of ^{32}P-UTP and other nucleotides as described in the materials and methods section. The newly transcribed RNAs were hybridized to a nitrocellulose membrane on which cDNAs of 3-β-HSD (20 μg), β-actin (20 μg) and vector (20 μg) have been immobilized. Results were recorded by autoradiography for visual display (*upper*) and quantified directly from the membranes by phosphorimage (*lower*). Values in (A) and (B) are the mean ± SE of triplicates from a representative experiment. Three separate experiments were performed.

30-min incubation with substrate DHEA, the major metabolite formed is androstenedione with only a minor conversion to testosterone (~1%), and no other metabolites were detected. 3β-HSD activity is defined as the metabolic generation of total products derived from substrate. In the calculation, the contributions of all the products observed, androstenedione + testosterone, were accounted for as derived form the conversion of DHEA to Δ_4-metabolites by 3β-hydroxysteroid dehydrogenase. To study temporal changes of 3β-HSD activity following *in vivo* treatment with 2.5 μg of hCG, substrate DHEA (1 μM ^{14}C DHEA + 10 μM DHEA, unlabeled), was added to the Leydig cells following preincubation with inhibitors aminogluthetimide (as above) and incubated for 30 min. Steroids in the medium were analyzed as indicated above. In other experiments, pregnenolone (1 μM ^{14}C-pregnenolone [55.4 mCi/mmol, DuPont NEN] + 10 μM pregnenolone, unlabeled) was added as substrate, after preincubation of the cells with aminogluthetimide (as above) and 10^{-5} M spironolactone for 15 min. The latter was previously shown to effectively inhibit CYP17 in Leydig cells (10). The cells then were incubated for 30 min in presence of substrate. Steroids in the medium were processed, and results evaluated as indicated above.

LH receptor binding study

For these studies, we used highly purified hCG (CR121) kindly provided by Dr. G. Bialy (Center for Population Research, NICHD). hCG was enzymatically radiolabeled with ^{125}I (21), followed by purification on Sepharose Concanavalin as previously described (22). To release endogenous bound hCG for determination of total receptor (free and occupied), Leydig cells were pretreated with 2 ml of ice-cold 50 mM glycine-HCl buffer containing 100 mM NaCl (pH 3) for 2 min at 4 C. Reactions were stopped by adding 10 ml Medium 199 with 0.1% BSA (4 C) and immediately centrifuged at 1,500 × *g* for 10 min at 4 C. LH/hCG receptors were then determined by incubation of Leydig cells with labeled hormone ^{125}I-hCG (8×10^4 cpm/0.5 ng; specific activity: 34.2 μCi/μg) and cold hCG (20 ng) in 0.1 ml of Medium 199/0.1% BSA. Nonspecific binding was measured in the presence of 100 IU Pregnyl. All incubations were performed for 18 h at 4 C in a final volume of 1 ml Medium 199/0.1% BSA. Binding was terminated by adding 2 ml of ice-cold PBS followed by centrifugation at 3,000 × *g* for 15 min at 4 C. The supernatant solutions were aspirated and discarded. Cell pellets were further washed twice with 2 ml of cold PBS. After aspiration of the supernatants, the cell bound radioactivity was determined by automatic γ-spectrometer.

All experiments were performed at three times in triplicate unless specified. Results are the mean ± SE. Statistical significance was evaluated by ANOVA followed by Duncan's multiple range test.

Results

Regulation of 3β-HSD mRNAs detected by differential display screening and verified by RNase protection assay in gonadotropin-desensitized Leydig cells

Previous studies have demonstrated that testosterone production and LH receptor were considerably inhibited in gonadotropin-desensitized Leydig cells. To elucidate the components contributing to this inhibition, we attempted to screen expressed genes that might be participating in this regulatory mechanism using mRNA Differential Display method. Different combination of primer sets composed of 5 anchor primers and 20 arbitrary 10-mers were used for mRNA amplification by RT-PCR. Several of the PCR fragments employed showed obvious changes between the group treated with desensitizing dose of hCG and nontreated control group. Analysis of sequences of the PCR fragments using the FASTA program to search GenBank and EMBL databases revealed that two of the clones amplified by primers of 5′- T_{11}AA-3′ and 5′-GATCAAGTCC-3′, were identical

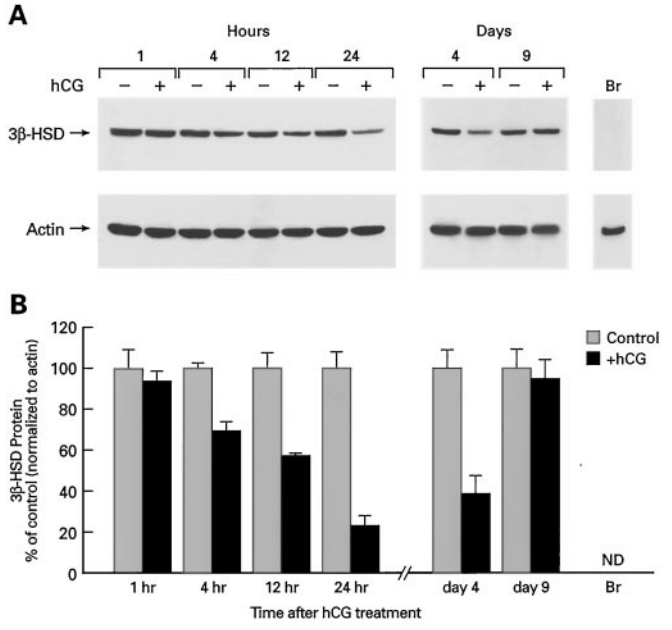

FIG. 5. Down-regulation of 3β-HSD protein by hCG in desensitized Leydig cell. Leydig cells were obtained and purified from male rats at different times after sc injection of PBS alone (vehicle control), or with 2.5 μg of hCG. Total cell proteins were separated (50 μg/well) on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were probed with antibody against 3β-HSD and reprobed with actin antiserum (see *Materials and Methods*). The individual immunoreactive signals were detected by ECL system in x-film and quantitated by densitometry. Three separate experiments were performed and each experiment analyzed by Western blots (triplicates). A representative Western blot of one such experiment is shown above with bands for 3β-HSD and actin at the times indicated, and below are shown results mean ± SE from three separate experiments. Values normalized to actin are plotted as per cent from controls at the individual times. Brain (Br) was used as a negative control for 3β-HSD. Values at 1 h and 9 days were not different from controls. At 4, 12, and 24 h and 4 days, values were significantly different from controls (see results). There were no significant differences among controls at the different times.

to the 3′-end noncoding region of 3β-HSD type I and type II cDNA (Fig. 1A, *lower panel*). As shown in Fig. 1A, both types (100 bp for type I and 86 bp for type II) of 3β-HSD mRNAs were down-regulated by the desensitizing dose of hCG for all three sets of RNA samples independently prepared at different times.

RNase protection assays verified the down-regulation of the two types of 3β-HSD mRNAs induced by *in vivo* treatment with gonadotropin. Two sets of primers were designed (Fig. 1B, *lower panel*): P0 and PI for type I, and P0 and PII for type II, to amplify the fragment in the coding region of 3β-HSD mRNAs by RT-PCR. The sequences of the PCR cloned products (216 bp) were identical to the type I and type II cDNAs of 3β-HSD as expected. RNase protection assays showed that the density of ^{32}P-labeled protected DNA-RNA hybridized fragments (216 bp) for both type I and type II (Fig. 1B) are considerably lower in the hCG-treated samples than in the nontreated samples, whereas the protected bands (290 bp) for β-actin (internal control) are very similar in all samples. The shorter protected fragments (<180 bp), indicated by *dash line arrow* in Fig. 1B, *upper panel*, correspond to the type II mRNA in the assay with the type I cRNA probe and similarly for the type I mRNA when using the type II probe. These shorter protected fragments were generated by digestion with RNases due to the nucleotide difference between the fragments corresponding to the two types of 3β-HSD (Fig. 1B, *lower panel*).

Dose dependency of 3β-HSD mRNAs regulation

Previous studies have demonstrated that in contrast to desensitizing doses of hCG (1–2.5 μg), lower doses (2–100 ng) stimulated testosterone production (10). To examine whether the induction of testosterone by low doses of hCG was related to 3β-HSD, the effects of 10 and 100 ng hCG doses were compared with those of a desensitizing dose (2.5 μg) of hCG. RNase protection assays confirmed that the mRNAs for both types of 3β-HSD were down-regulated 5- to 8-fold by the higher dose (2.5 μg) of hCG [$P < 0.001$], Fig. 2, *lower panel*, whereas neither of the 3β-HSD isoforms was increased by the lower doses (10 and 100 ng) of hCG. On the contrary, the type I mRNA showed minor but significant [$P < 0.01$] down-regulation by the lower doses of hCG, whereas the type II showed no significant change [$P > 0.05$] with 10 and 100 ng of hCG when compared with the nontreated sample (Fig. 2, *lower panel*).

Temporal study of 3β-HSD mRNAs regulation by hCG

Temporal profiles of 3β-HSD mRNA (Fig. 3) showed that down-regulation for type I enzyme preceded (within 1 h after the treatment of hCG) the type II enzyme (1–4 h). Four hours after treatment, the mRNA of both enzymes was reduced by 40%. Maximal mRNA reduction was observed for both 3β-HSD forms at 24 h. The type I mRNA returned faster (at day 4) to the control levels than the type II enzyme (at day 9). The reduction of 3β-HSD mRNA [$P < 0.01$] preceded the down-regulation of LH receptor [1 and 4 h (3β-HSD I and II) *vs.* 12 h (LH receptor binding)]. The maximal inhibition of receptor binding was as that of 3β-HSD at 24 h and the nearly complete restoration of LH receptor binding activity was observed 9 days after treatment (80% of control levels, Fig. 3A). Thus, recovery of receptor binding activity occurred later than that observed for 3β-HSD type I but was comparable with the type II enzyme.

Down-regulation of 3β-HSD is at the transcriptional level

To discern whether the down-regulation of 3β-HSD mRNA was due to increased degradation or decreased transcriptional activity, Leydig cells from rats injected with a desensitizing dose of hCG were incubated with actinomycin D to block mRNA synthesis. Subsequently, Northern blot analysis was used to monitor degradation rate of the mRNA. Significant reduction of the 3β-HSD mRNA (1.7 kb) of cells from animals treated with hCG was observed at all times after Actinomycin D treatment (Fig. 4A). The rates of degradation were very similar for the two groups with half-lives of 5.1 and 5.3 h for the hCG-treated and nontreated groups, respectively. Nuclear run-off assays demonstrated that the down-regulation was caused by reduction of transcriptional activity. Figure 4B shows that newly synthesized mRNA was

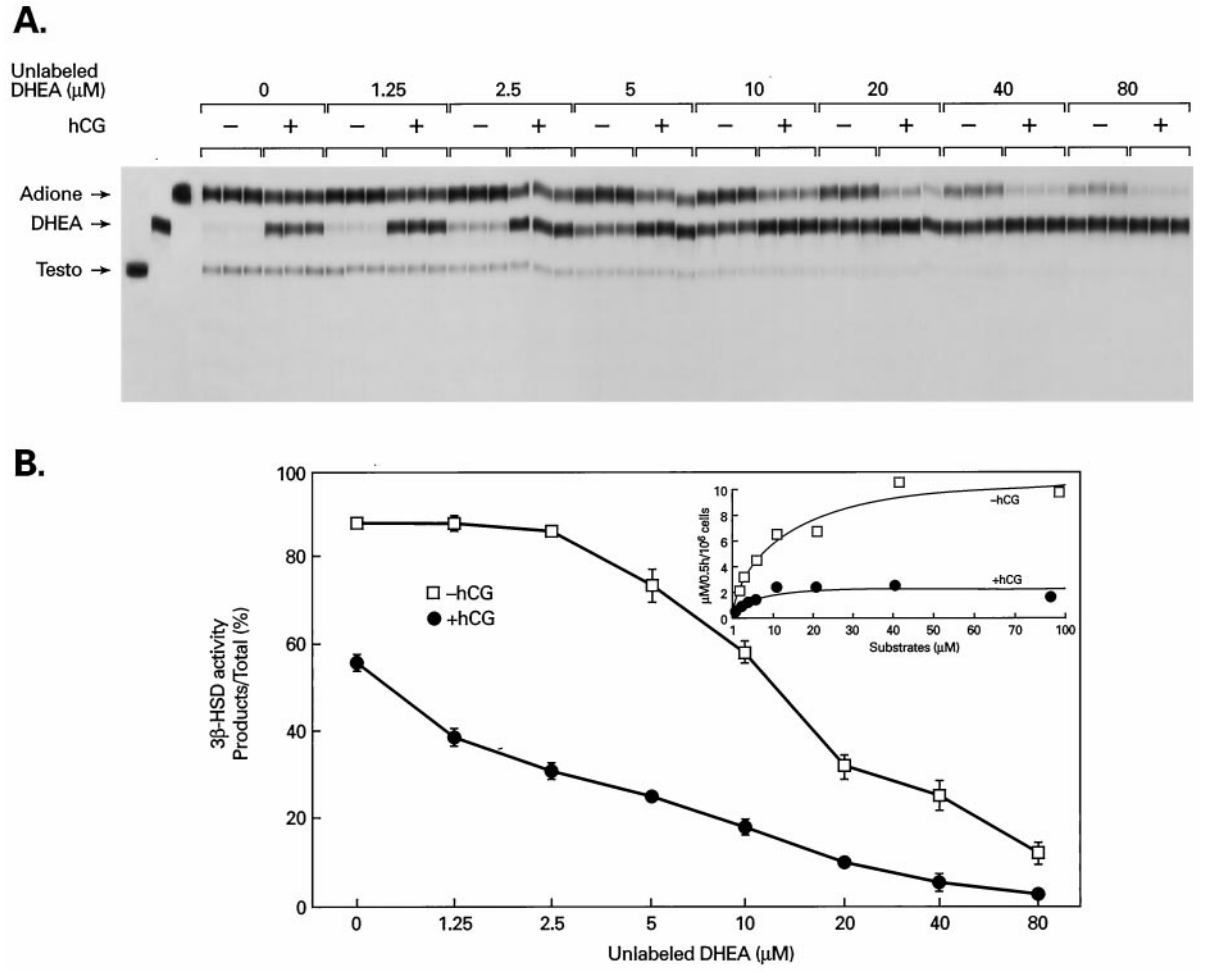

FIG. 6. Down-regulation of enzymatic activity of 3β-HSD by hCG in desensitized Leydig cell. Leydig cells were obtained and purified from male rats 24 h after sc injection with and without 2.5 μg of hCG. Intact Leydig cells (1×10^6) after preincubation with aminoglutethmide (100 μg) for 15 min were immediately incubated with 1 μM substrate of [^{14}C]-DHEA in medium 199 with 0.1% BSA and different concentrations of unlabeled DHEA at 37 C for 30 min. [^{14}C]-DHEA and [^{14}C]-androstenedione (A-dione) were separated by TLC, signals were recorded by autoradiography for visual display (upper panel) and TLC were quantitated by phosphoimager analysis (*lower panel*). A representative experiment mean ± SE of triplicates is shown. Three separate such experiments were performed. 3β-HSD activity is defined as metabolic generation of total products (Androstenedione + Testosterone) from conversion of substrate DHEA and expressed as percent of control (see also *Materials and Methods*).

significantly reduced ($P < 0.001$) in the hCG-treated Leydig cell when compared with the untreated control.

Down-regulation of 3β-HSD protein by hCG in Leydig cells

Western blot analyses revealed that the rat Leydig cell 3β-HSD protein (42K) was down-regulated by a desensitizing dose (2.5 μg) of hCG (Fig. 5). A significant reduction of 3β-HSD protein was detected in immunoblots 4 and 12 h after treatment by 31 ± 2% and 41 ± 2% from control, respectively (mean ± SE, n = 3 separate experiments in triplicate) ($P < 0.01$). A further reduction of 3β-HSD was found at 24 h ($P < 0.001$), and no additional decrease was observed at 4 days after hCG treatment. The protein level returned at 9 days to values that were no different from controls.

Enzymatic activity of 3β-HSD in Leydig cells

The conversion of Δ5-DHEA to Δ4-androstenedione was significantly reduced in the intact Leydig cells from hCG-treated animals when compared with nontreated controls at all concentration of DHEA [$P < 0.001$] (Fig. 6). The maximal enzymatic activity (kcat) was reduced by 4-fold from 11.3 ± 0.68 μM 0.5 $h^{-1}/10^6$ cells (control) to 2.4 ± 0.86 μM 0.5 $h^{-1}/10^6$ cells (hCG-treated group) ($P < 0.001$). The relative enzymatic activity ($kcat/K_m$) was also reduced by 2-fold [(1.16 ± 0.19 *vs.* 0.66 ± 0.09, [$P < 0.001$)]. The time-course study of 3β-HSD activity showed reduction of enzyme activity at 4 h after hCG treatment with substrate DHEA [$P < 0.001$] (Fig. 7, A and B), or pregnenolone [$P < 0.05$] (Fig. 7, C and D). Further decreases in enzyme activity were observed at 12 and 24 h in hCG treated animals compared with control and treatment at 4 h ($P < 0.001$) (Fig. 7). No significant changes at 1 h after treatment with hCG were observed. The enzymatic changes were consistent with those observed at the protein level (Fig. 5) but occurred after the down-regulation of type I mRNA (at 1 h, Fig. 3B), whereas they were concomitant with the change of type II mRNA (at 4 h, Fig. 3C).

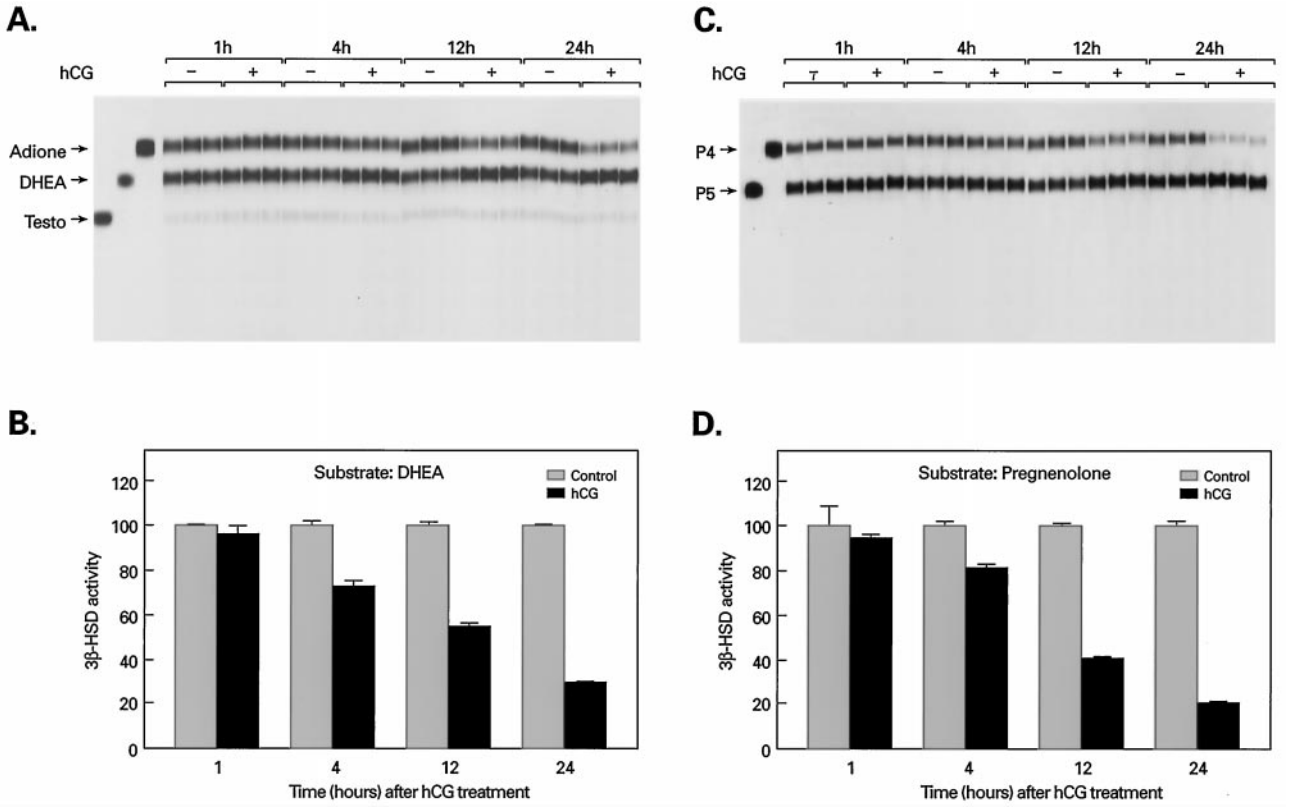

FIG. 7. Time course study of 3β-HSD enzymatic activity regulated by hCG. Leydig cells were obtained from male rats injected sc with vehicle alone or 2.5 μg of hCG at different times (1–24 h) after the treatment. Intact Leydig cells (1×10^6) following a 15-min preicubation with aminoglutethimide, were immediately incubated with 1 μM substrate of [^{14}C]-DHEA in medium 199 with 0.1% BSA and 10 μM unlabeled DHEA at 37 C for 30 min. The [^{14}C]-DHEA and [^{14}C]-androstenedione (A-dione) were separated by TLC and radioactivity from products and substrate were recorded by autoradiography for visual display (A). Steroids standards (^{14}C labeled) are shown left of autoradiograms (Adione, androstenedione; Testo, testosterone; DHEA, dehydroepiandrosterone). TLCs were quantified by phosphorimager analysis (B). Quantitation of products was performed as described in *Materials and Methods*. In experiments when substrate pregnenolone was used (1 μM ^{14}C pregnenolone +10 μM unlabeled pregnenolone) (C and D), cells were preincubated with spironolactone in addition to aminoglutethimide. Subsequent incubation and analytical and quantitation steps were as for DHEA (see above, and *Materials and Methods*). Steroids standards (^{14}C labeled) are shown right of the autoradiogram (P4, progesterone; P5, pregnenolone). 3β-HSD is defined as metabolic generation of total products from conversion of substrate DHEA or pregnenolone and expressed as percent of the control at the individual times indicated. Four separate experiments were performed with substrate DHEA, and two experiments with substrate pregnenolone. Representative experiments, mean ± SE of triplicates are shown.

All of the above changes occurred before the down-regulation of LH receptors (12 h, Fig. 3A).

Differential regulation of 3β-HSD mRNA by the desensitizing dose of hCG in rat Leydig cell and luteinized ovary

Types I and II are also the two major isoforms for 3β-HSD in ovary (4), and play a key role in the synthesis of steroid hormones. Like in testis, the LH/hCG receptor down-regulation and gonadotropin desensitization was induced by treatment of pseudopregnant animals with high doses of hCG (12, 23). To determine whether the 3β-HSD mRNAs are regulated by hCG treatment, we analyzed the mRNA of luteinized ovaries from pseudopregnant rats treated or untreated with a single desensitizing dose of hCG by RNase protection assay. Both type I and II mRNAs (216 bp protected band) of 3β-HSD are not regulated by hCG in luteinized ovary; the 3β-HSD mRNAs in Leydig cell, however (used as a positive control) displayed marked down-regulation by hCG treatment (Fig. 8). The internal control of β-actin (290 bp) showed no change in all samples.

Discussion

These findings have demonstrated that desensitizing doses of hCG cause a marked inhibition of 3β-HSD gene expression in the Leydig cell. The 3β-HSD mRNA (type I and II), the enzyme protein, and its enzymatic activity, were significantly down-regulated by the administration of a single dose (2.5 μg) of hCG. The hCG-induced reduction of 3β-HSD activitiy resulted from inhibition of the expression of Leydig cell mRNA isoforms I and II. The impaired conversion from Δ5 to Δ4 steroids contributes to the decreased *in vitro* testosterone responses to hCG observed in Leydig cells from animals treated with high doses of hCG (8, 10). The half-life of 3β-HSD mRNA was unchanged by hCG treatment, suggesting that the down-regulation of 3β-HSD transcripts was not due to mRNA degradation but rather to changes at the transcriptional level. This was confirmed by

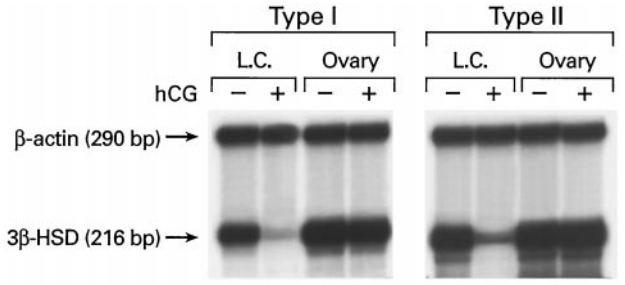

FIG. 8. Differential regulation of 3β-HSD mRNA by a desensitizing dose of hCG in rat Leydig cells and luteinized ovary analyzed by RNase protection assay. Total RNA samples (10 μg) from Leydig cells and luteinized ovary treated with and without desensitizing dose of hCG (2.5 μg) were hybridized to the ^{32}P-labeled type I or type II of 3β-HSD together with β-actin (internal control). Protected fragments resolved on a denatured sequencing gel were recorded by autoradiography for visual display and protected fragments in the gels were quantified by phosphoimager analysis. The decrease in 3β-HSD observed in the Leydig cells was commensurate with results observed in Fig. 3, at 24 h. No changes between control and hCG treated animals were observed in ovarian samples. Three separate experiments in duplicate were performed. A representative experiment is provided in this figure.

nuclear run-off assays, which showed that newly synthesized 3β-HSD mRNA was significantly reduced in Leydig cell nuclei from hCG-treated animals.

The inhibitory effect of gonadotropin on 3β-HSD transcription could be related to negative feedback by the initial elevation of intracellular steroids during the acute stimulatory effect of the hormone. Such inhibition could be exerted by metabolic products of androgen metabolism, either testosterone or estrogen, or by the influence of precursors of this pathway such as progesterone. Testosterone is the major steroid produced and secreted by the gonadotropin-stimulated Leydig cell. There is existing evidence of an *in vitro* inhibitory effects of endogenous (induced by cAMP) and exogenous androgen on 3β-hydroxysteroid mRNA (24, 25), enzyme activity, and enzyme mass (25) from studies employing acute mouse Leydig cell cultures (24, 25). In both studies, the decrease of 3β-HSD caused by androgen was prevented by an inhibitor of steroid synthesis (24) or by an androgen receptor antagonist (25).

Estradiol, a metabolic product of androgen metabolism, and estrogen receptors are also present in the Leydig cell (9). Although estradiol levels are low compared with those of testosterone, they have been found to mediate inhibitory actions on 17α-hydroxylase/17–20 desmolase (CYP17) mRNA and enzymatic activity after treatment with high doses of hCG (8, 26, 27). The accumulation of steroid precursors due to the marked reduction of CYP17 was prevented by administration of the antiestrogen, tamoxifen, before treatment with hCG (8, 27). In the ovary, estradiol and progesterone are the major steroid products, and testosterone levels are very low due to the preferential conversion of its precursor, androstenedione, to estrogen by aromatase (2, 28, 29). In addition, we have demonstrated a lack of regulation of ovarian 3β-HSD by gonadotropin, in contrast to the inhibition observed in the Leydig cell. Therefore, the participation of estradiol or progesterone in regulation of Leydig cell 3β-HSD is unlikely.

The above evidence, specifically the androgen inhibitory effects *in vitro* (24, 25) and the lack of modulation of 3β-HSD *in vivo* by gonadotropin in the ovary (this study), indicates that androgen produced acutely by the hCG stimulus *in vivo* is the most likely candidate to exert direct or indirect effects on the enzyme's gene transcription. However, there are major temporal differences between the inhibitory action of the androgen on 3β-HSD observed in mouse Leydig cells cultured *in vitro* (at 3–6 days) (25), and the rapid changes in enzyme parameters induced by hCG *in vivo*, which are evident within a few hours (this study). This indicates that additional or different mechanism(s) may be operative in the regulation of rat 3β-HSD *in vivo*, or alternatively may merely reflect differences in species (rat *vs.* mouse) and/or experimental conditions (*in vivo vs. in vitro*).

The rat 3β-HSD promoters have not yet been cloned, and the human gene I (30, 31) and gene II (GeneBank/EMBL, Accession number M77144) promoters do not contain estrogen, androgen, or other steroid consensus responsive elements within their 5′ flanking regions. However, the half-site of the specific androgen response element (GGTTCT) (32, 33) is present in type I and II 3β-HSD. By extrapolation from the human gene, it is possible that gonadotropin treatment inhibits 3β-HSD transcription through nuclear actions of steroids, either dependent (34) or independent of DNA binding (35, 36), or by other mediators of hCG action. Alternatively, gonadotropin action could inhibit the transcription of a yet unidentified gene product (*e.g.* transcription factor) that is required for expression of the enzyme, or hormone treatment may cause the activation of an inhibitor of the enzyme's transcription.

The two types of 3β-HSD genes differed in their hCG-induced down-regulation, and recovery. Down-regulation of the type I enzyme preceded that of the type II enzyne (1 *vs.* 4 h, Fig. 3) Furthermore, although both 3β-HSD mRNA isoforms are reduced to equivalent levels following 1 day of treatment, recovery occurred more rapidly for the type I enzyme than for the type II enzyme. The recovery of the type II enzyme is coincident with the temporal pattern of the recovery of LH receptors, indicating the existence of differential regulation among the 3β-HSD isoforms following hCG action.

RNAse protection assay showed that both types of 3β-HSD mRNA are highly expressed in the testis and ovary. However, down-regulation of the enzyme is only observed in the testis, indicating that tissue-specific factors probably affect transcription in the male gonad. These studies have demonstrated that the marked reduction of progesterone production in response to hCG *in vitro* previously observed in luteal cells from desensitized animals (12) is not related to changes in 3β-HSD, but probably to impairment of early steps in the biosynthetic pathway.

These studies have revealed that 3β-HSD does not contribute to the positive regulation of androgen production observed in previous studies in Leydig cells of animals treated with a single near-physiological concentration of hCG (7–9). In contrast, minor but significant down-regulation of 3β-HSD type I was induced by low doses of hCG (0.01 and 0.1 μg), but no significant changes were observed in the type II isoform. The down-regulation of both 3β-HSD mRNA isoforms by high doses of hCG preceded the receptor down-regulation indicating that down-regulation of the enzyme

was not due to the reduction in receptors (Fig. 3). Rather, the available data on enzymatic down-regulation point to early events in gonadotropin action that lead to marked regulation of steroidogenic enzymes [3β-HSD, this study, and CYP17 (37, 38)] and subsequently of gonadotropin receptors. Our early studies demonstrated significant accumulation of pregnenolone in Leydig cells of animals treated with a desensitizing dose hCG, whereas nondetectable levels were observed in cells of untreated control animals (10). This could reflect inhibition of the 3β-HSD parameters observed in this study, and a contribution of the enzyme to the inhibition of androgen production in gonadotropin-induced desensitization of Leydig cells (10). Whether the negative changes in steroidogenic enzymes and receptors induced by *in vivo* treatment with hCG are related to a single master switch, or to a series of steps, remains to be determined.

In conclusion, this study has demonstrated that the metabolic step of steroid metabolism that is governed by 3β-HSD was down-regulated by a desensitizing dose of hCG in the Leydig cell, but not in the luteinized ovary. This steroidogenic lesion can contribute to the gonadotropin-induced inhibition of androgen production, and is attributable to inhibition of transcriptional regulation of the enzyme secondary to gonadotropin action.

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