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Effect of Leptin on CYP17 Enzymatic Activities in Human Adrenal Cells: New Insight in the Onset of Adrenarche*

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ABSTRACT

CYP17 is a microsomal enzyme embodying two distinct activities, 17α-hydroxylase and 17,20-lyase, essential for the synthesis of cortisol and sex hormone precursors, respectively. The two activities are differentially regulated in a tissue and developmental stage-dependent fashion. Leptin might play a role in such differential control. Low dose leptin caused a significant increase in 17,20-lyase activity in adrenal NCI-H295R cells expressing leptin (OB) receptor (OB-R), without significant sustained influence on the 17α-hydroxylase activity. To analyze the time dependence of this leptin effect, the impact of long and short-term leptin treatment was studied. To assess the relationship with the OB-R signal transduction pathway, the same experiments were performed in intact cells and in a reconstituted system. The long- and short-term studies in intact cells and in microsomes suggest that the 17α-hydroxylase activity of CYP17 can be promptly stimulated by leptin, but that the effect is transient. In contrast, physiological doses of leptin steadily enhance 17,20-lyase activity. This influence is direct, OB-R specific and dependent on the integrity of the signal transduction pathway. The 17,20-lyase activity stimulation relies on phosphate incorporation, as demonstrated by the loss of leptin-dependent 17,20-lyase stimulation after phosphate removal, and by the fact that the DHEA production appears to be related exclusively to the presence of phosphorylated CYP17, independently from novel protein synthesis. The mechanism underlying the observed events seems to involve CYP17 phosphorylation, a feature of the OBR signal transduction pathway, and a process already shown to be crucial for 17,20-lyase activity. (*Endocrinology* **141:** 1446–1454, 2000)

ADRENARCHE OCCURS EXCLUSIVELY in primate species, in children typically around 8–9 yr of age. It correlates histologically with the maturation of the *zona reticularis* within the adrenal cortex and is independent from pubertal development. Endocrine hallmarks of adrenarche are the increasing circulating levels of DHEA, DHEAS, and androstenedione. Such increase appears to be due to an enhancement of the 17,20-lyase activity of CYP17. The physiological factor(s) involved in this differential regulation and their interaction are still unclear.

Among several possible factors, leptin deserves particular notice. Leptin, the product of the *ob* gene, is an adipocyte-derived peptide hormone that exerts major effects on energy homeostasis. In addition, it affects reproductive function in animals (1, 2) and humans. Patients lacking leptin or its receptor show no signs of sexual maturation at the expected time of puberty (3, 4). In normal boys and girls, leptin increases some time before the gonadotropin peak, and its variations appears to be independent from changes in auxological parameters, mainly in boys. (5). These data suggest a permissive action of leptin for the initiation of sexual development. Interestingly, the rise in leptin levels occur at age 8–10, roughly corresponding to the time of adrenarche.

The clinical and experimental data on the relationship between leptin and adrenal maturation at the time of adrenarche are controversial. Although some authors failed to observe a relationship between DHEAS levels and leptin during pubertal development in normal boys (6), others reported a positive correlation between DHEA and leptin in patients with the Prader-Labhart-Willi syndrome, independently from body mass index (BMI). (7). Recent experimental studies demonstrated a direct inhibition of cortisol release by leptin in bovine (8), human, and rat (9) adrenal cells in culture, although at supraphysiological doses (100–1000 ng/ml, normal adult plasma levels 16.9 ± 10.9). The mechanism underlying such reduction seems to be a decrease in CYP17 messenger RNA (mRNA) accumulation. No data are available concerning a direct effect of leptin on CYP17 enzymatic activities. We therefore decided to investigate this aspect of CYP17 activity regulation in NCI-H295R adrenocortical carcinoma cells.

Materials and Methods

Reagents

Human recombinant leptin was obtained from Peprotech (London, UK). All cell culture reagents are purchased from Life Technologies, Inc.. Cholesterol, 22R-hydroxycholesterol (22R-HC), 8Br-cAMP were purchased from Sigma (St. Louis, MO).

Cell culture

Human adrenocortical carcinoma cells NCI-H295R (ATCC No. CRL-2128) and human ovarian adenocarcinoma cells NIH OvCar3 (ATCC No. HTB-161) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by the purchaser. For the initial plating, 7.5×10^4 cells were seeded on 35-mm plates. Medium was replaced every 24 h. Experiments were performed

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48 h thereafter (confluence about 70%). Leptin treatment was then initiated at the given concentrations and discontinued at the given time points. Because most cells detached from the culture plate under leptin treatment, the medium was collected, and the detached cells were harvested by centrifugation and replated. The cells that remained adherent were trypsinized and collected by centrifugation. The viability of the detached cells was controlled by trypan blue exclusion.

For all the following experiments, the cells expressing OB-R (see later) were selected by treatment with 30 pM leptin for 24 h, collected by centrifugation, and replated in the absence of leptin. These cells were normally reattached after 12 h. Upon further treatment with leptin, the selected OBR+ cells detached again, and the following experiments were conducted in suspension. To assay enzyme activity and phosphorylation state of CYP17, such selected cells were used, unless otherwise indicated. As further characterization of the populations of NCI-H295R cells, and to check the specificity of leptin effect on CYP17, the classical stimulator 8Br-cAMP (200 μM) was added to the cells either 2 h prior or simultaneously with the addition of steroid precursors. The expected products (17OH-progesterone and DHEA) were then measured in cell extracts (see later).

For the experiments where the progesterone production was investigated, the cells were plated at a concentration of 50×10^3 cells/well in 96-well plates and cultivated for 24 h in medium plus serum, and consequently selected for OBR using leptin for 6 h. The stimulation was performed using 200 μM 8Br-cAMP, 30 pM leptin, and 50 μM cholesterol or 22R-HC, for 30′, 6 and 2 h, respectively.

Protein synthesis inhibition

OBR + and OBR− cells were separated by preincubation with 30 pM leptin for 6 h. Two hours before the addition of the protein synthesis inhibitor cycloheximide (Sigma), steroidogenic precursors were added as described below. At time 0 min, 40 μM cycloheximide and 30 pM leptin were added to NCI-H295R cells with or without 0.1 mCi/ml ^{35}S-methionine or ^{32}P orthophosphate (^{32}Pi) (200 μCi/ml). The reaction was stopped by removing the medium and lysing the cells at times 2 h 30 min, 4 h 30 min and 6 h 30 min. Enzyme activity and immunoprecipitable CYP17 labeled protein content were analyzed as described below. Western blot analysis was performed using standard procedures.

RNA analysis: RT-PCR and Northern blot

Total RNA was extracted from detached and attached cells in the absence or presence of 30 and 100 pM leptin (24 h treatment) using the RNeasy minikit (QIAGEN, Hilden, Germany). RT was performed using superscript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). PCR amplification of OBR exons 15–17 was performed as described (4). PCR products were separated by electrophoresis on 1% agarose gel and Southern blot was performed following standard procedures. A α-^{32}P-dATP labeled human OB-R complementary (cDNA) was used as hybridization probe. PCR amplification of human StAR, CYP11A1, and adrenodoxin (ADX) was done using the following primers and cycling conditions: StAR sense 5′-GCAGCAGCAGCGGCGGCAGCAG-3′ (designed to distinguish between the active gene and the pseudogene); antisense 5′-ATGAGCGTGTGTACCAGTGCAG 3′; 94 C for 45 sec, 64 C for 30 sec, 72 C for 60 sec × 30 cycles; CYP11A1 sense 5′-TACCAGAGACCCATAGGAG-3′, antisense 5′-CGCACACCCCAGCCAAAGC-3′; 94 C for 45 sec 58 C for 45, 72 C for 60 sec × 30 cycles; ADX sense 5′-CTGCCGCCCCCGCCTCTT-3′, antisense 5′-CAGTCTGAACACATAGCTT-3′, 94 C for 45 sec, 58 C for 45 sec, 72 C for 60 sec × 30 cycles. For human cytochrome b_5 amplification the primers and PCR conditions were as follows: sense 5′-TGGCAGAGCAGTCGGACG-3′, antisense 5′-GCTCTTCCTGCGCTGACTTCTG-3′, 94 C for 45 sec, 52 C for 60 sec, 72 C for 60 sec × 40 cycles. Northern blot analysis was performed using standard procedures, hybridizing 20 μg of total RNA extracted from OBR+ NCI-H295R cells to a radiolabeled human CYB5 cDNA fragment (bp 1–460).

In vivo metabolic labeling and immunoprecipitation

Cells were metabolically labeled with either ^{32}P orthophosphate (^{32}Pi) (200 μCi/ml, NEN Life Science Products, Boston, MA) for1 h in phosphate-free medium (Life Technologies, Inc. 11963–022) or with ^{35}S-methionine (100 μCi/ml, NEN Life Science Products, easytag) for 2 h in methionine-free medium (Life Technologies, Inc.). Labeled cells were lysed in 1 × PBS, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton-X, 10% glycerol in the presence of protease inhibitors (phenylmethanesulfonyl fluoride 34 μg/ml, 0.7 μg/ml pepstatin, 5 μg/ml leupeptin, Roche Molecular Biochemicals) and phosphatase inhibitors for the phosphorylation experiments (100 mM sodium-fluoride, 10 mM sodium-pyrophosphate, 2 mM sodium-orthovanadate, Sigma). The lysates were clarified by centrifugation at 15,000 × *g* for 10 min. The supernatants were then bound to Protein A Sepharose (Amersham Pharmacia Biotech, Dubendorf, Switzerland) preincubated (at least 20 min at RT) with antihuman CYP17 antibodies (a generous gift from Prof. M. Waterman, Nashville, TN) at a dilution 1:50.000. The cell lysates were incubated on the Protein A Sepharose/Ab complex O/N at 4 C. The immune complexes were then extensively washed and analyzed on 10% SDS-PAGE. Quantification of ^{32}P and ^{35}S incorporation was conducted by cutting the bands and counting. The standardization was done by dividing the ^{32}P cpm and ^{35}S cpm per mg total protein. The experiments were repeated three times.

Enzyme assay

Intact cells. Steroidogenic precursors (progesterone for 17α-hydroxylase activity and 17OH pregnenolone for 17,20-lyase activity) were added at the concentration of 750 ng/ml after suspension in 1 × phosphate buffer. Six hours after addition, supernatant were removed for the time point 0 and leptin treatment was initiated. The same procedure was adopted for the several time points. All samples were kept frozen at −20 C until measured. To assay the intracellular concentration of steroids, cell lysates were prepared as described above, protein content was measured using Bio-Rad Laboratories, Inc. protein assay reagents, the steroids were extracted with ethyl acetate/isooctane (1:1) and assayed by RIA.

Microsomes (reconstituted system). Microsomal membranes were prepared from OBR + NCI-H295R cells, as described (10). The total protein content was 1.2 mg/ml. CYP17 activity was measured by incubating 10 μg of microsomal protein with 500 nmol of progesterone or 17OH-pregnenolone for 30 min, as previously described (10).

Long term. Thirty picomoles leptin were added at time 0 min, and incubations were discontinued at time 2, 4, 6, 10, 12, and 24 h thereafter, when cell extracts were prepared, steroid extracted and assayed in duplicate.

Short term. The experimental procedure is the same as described for the long-term experiments, with the exception of the time points: every 5 min over 1 h.

The alkaline phosphatase experiments were performed after 60-min leptin treatment (30 pM) in intact cells, subsequently lysed as previously described. The cell lysates were preincubated in ice-cold 50 μM Tris·Hcl, pH 8/1 mM, $MgCl_2$ at 37 C for 10 min. One unit alkaline phosphatase (Roche Molecular Biochemicals) (or an equivalent volume of water as control), was added and the reaction was stopped at time 0, 2, 4, 8, 12, 16, and 20 min with 50 mM EDTA. To assess more precisely the role of phosphate, 2 mM of the phosphatase inhibitor sodium-orthovanadate was added to the lysis buffer at each time point. The enzyme activity was measured at the several time points as described above.

The products were extracted with ethyl acetate/isooctane (1:1), concentrated by evaporation, resuspended in 500 μl RIA buffer (20 mM KH_2PO_4; 47 mM $Na_2HPO_4.2H_2O$; 5 mM NaN_3;1 g/liter bovine γ-globulin) and assayed by RIA.

Progesterone, 17OH-progesterone, and DHEA were measured in duplicates by RIA using Diagnostic Products Corp. kits (Los Angeles, CA).

Statistical analysis

All values, representing the results of three independent experiments, are expressed as mean ± SD and subjected to *t* test analysis (paired).

Results

The leptin treatment caused a dose-dependent detachment of NCI-H295R, NIH-Ov Car3, human hepatocarcinoma HepG2 cells, but no effect was observed in COS1 cells. About 80% of the cells detached already 1 h after initiation of treat-

ment, at very low leptin dosis (3 pM), with a maximum at 30 pM for 24 h (not shown). The detached cells were viable, as demonstrated by trypan blue exclusion test (not shown). Replating of the cells in the absence of leptin, led to complete reattachment. This phenomenon seems to be OB-R dependent because RT-PCR analysis of the human OB-receptor shows significant expression of OB-R only in those cells that detached upon leptin treatment (Fig. 1, A and B). That provided us with a selection assay for leptin responsive cells. The cells remaining adherent, expressing CYP17 but not expressing OB-R, were used as negative control. There was no significant qualitative time-dependent change in OB-R expression upon leptin addition in detached NCI-H295R cells (Fig. 1, C and D). Normalization of the RT-PCR data to expression of GAPDH gene (not shown) demonstrated that the differences in cDNA amount between the long- and the short-term treatments are due to variations in initial RNA content. Leptin treatment (30 pM for 24 h) caused a significant increase in 17,20-lyase in the OB-R+ NCI-H295R cells, but not on the OB-R- cells, and had no significant influence on the 17α-hydroxylase activity in both OB-R + or OB-R- cells (Fig. 2). Although the actual steroid product values differ between the secreted (medium) and the intracellular milieu, the general tendency appears to be the same, suggesting that there is an equilibrium between intra and extra-cellular steroids secretion, and that the increase of DHEA is not due to an alteration of the membrane permeability.

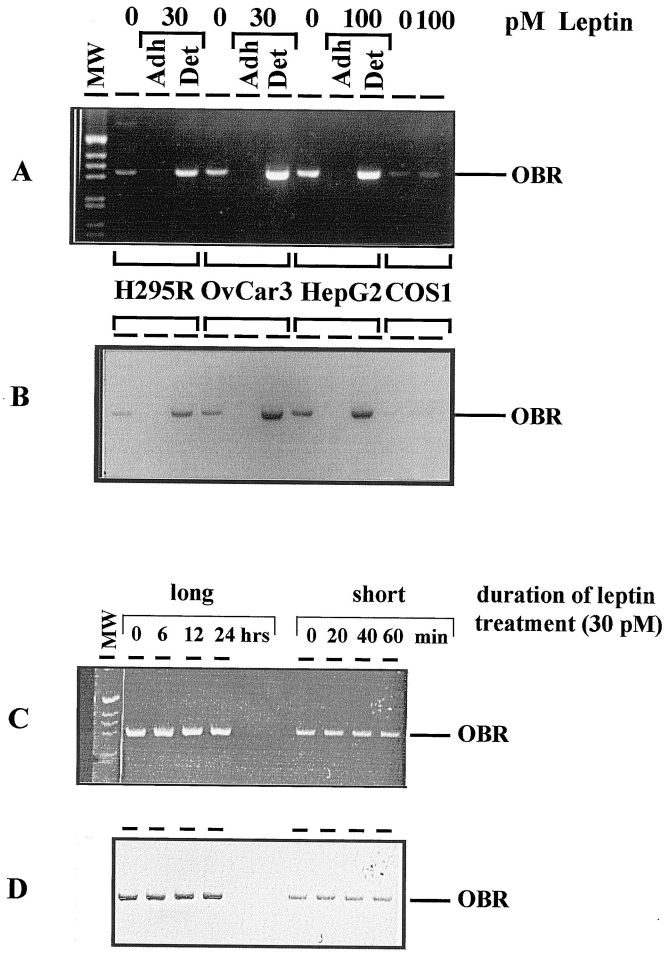

FIG. 1. Expression of human leptin receptor (OBR) in several cell lines. H295R, Human adrenal corticocarcinoma; OvCar3, human ovarian adenocarcinoma; HepG2, human hepatoma; COS1, monkey kidney. OBR is expressed only in those cells that detached under leptin treatment (A). The specificity of the amplified bands was confirmed by Southern blotting (B). COS1 and adherent cells do not express any OBR. No time-dependent change in OB-R expression in long- and short-term leptin treatment in detached NCI-H295R cells was observed (C). Again, the specificity of the amplified bands was confirmed by hybridization (D).

Addition of substrate up to 500 ng/ml did not saturate the enzyme, as is demonstrated by the absence of a plateau in the release of the products.

Additional evidence that the stimulatory effect of leptin on CYP17 enzymatic activity is specific and the OBR− cells are actually capable to respond to stimuli, is given by the demonstration that the classical stimulator of CYP17, cAMP, is able to significantly enhance 17α-hydroxylase and 17,20-lyase activities in both OBR+ and OBR− cells : 17OH-progesterone goes from 100 ± 5 to 403 ± 10 in OBR + and from 110 ± 10 to 390 ±7 ng/mg protein in OBR−cells; DHEA in OBR + cells: basal 75 ± 4 ng/mg protein, stimulated 350 ± 7 ng/mg protein; in OBR−cells: basal 68 ± 6 ng/mg protein *vs.* stimulated 345 ± 12 ng/mg protein. All the differences are significant: $P < 0.0001$. The time of addition (−2 h or time 0) had no significant influence on the outcome.

To analyze the time dependence of leptin effect, a long- and short-term leptin treatment was carried out. To assess the involvement of the OB-R signal transduction pathway in the observed events, the long- and short-term experiments were performed in intact cells and in a microsomal protein preparations in a reconstituted system.

17α-hydroxylase activity

Intact cells. As shown in Fig. 3, A and B, although in living cells an apparent effect of leptin on 17OH-progesterone production was detectable after 4 h, this effect disappeared in the following time points. *Microsomes* (OBR+): Upon short-term leptin treatment, 17OH-progesterone production showed a biphasic stimulation pattern, with a rapid initial increase (25–30 min) followed by a plateau and a steeper raise in the later time points. No elevation of 17OH-progesterone was seen in the absence of leptin (Fig 3C). Longer treatment appears to have an inhibitory effect on 17α-hydroxylase activity, although with an alternating pattern (Fig. 3D). The same trend was seen in the absence of leptin (Fig. 3D), suggesting an unspecific mechanism maybe related to cellular cyclic processes. The long- and short-term studies in intact cells and in reconstituted system thus suggest that the 17α-hydroxylase activity of CYP17 can be promptly stimulated by leptin, but that the effect is of short duration because already 10 h after initiation of treatment, the 17α-hydroxylase activity had returned to baseline (3B). This stimulation appears to be dependent on the presence of leptin in microsomes of OBR+ cells (Fig. 3, C and D), and/or of its receptor in intact cells, only when the treatment is kept long enough (2 h) (Fig. 3B). On the other hand, the 17α-hydroxylase activity seemed to be enhanced independently from this signal transduction pathway (Fig. 3, C and D).

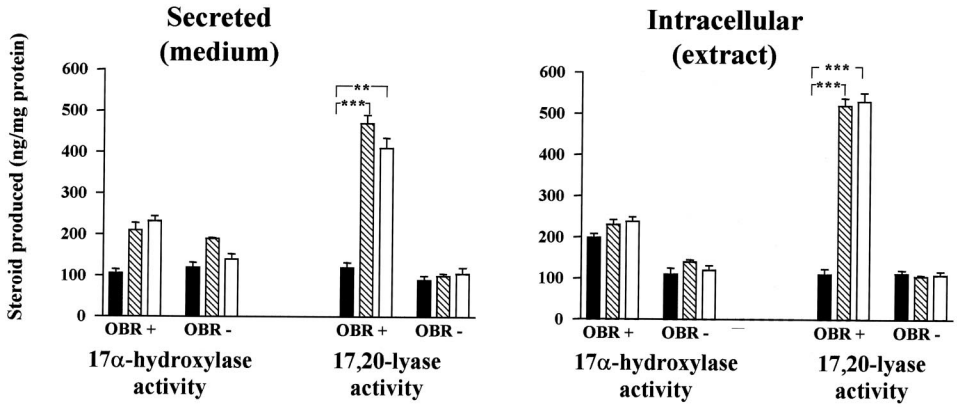

FIG. 2. CYP17 enzymatic activity assay in NCI-H295R cells in response to leptin treatment. 17/20-lyase activity was significantly stimulated by leptin treatment already at low doses (30 pM, *hatched bars*) only in OBR+ cells. No significant influence of leptin was seen in 17OH-progesterone production (17α-hydroxylase activity). The amounts of secreted steroids are comparable with those detected in the cell extract, suggesting the existence of an equilibrium between intracellular and extracellular product content. All values are expressed as mean ± SD and represent the results of three independent experiments. The data were subjected to *t* test analysis (paired), and only the significant difference are depicted: **, $P < 0.01$; ***, $P < 0.005$. Leptin: ■, 0 pM; ▨, 30 pM; □, 100 pM.

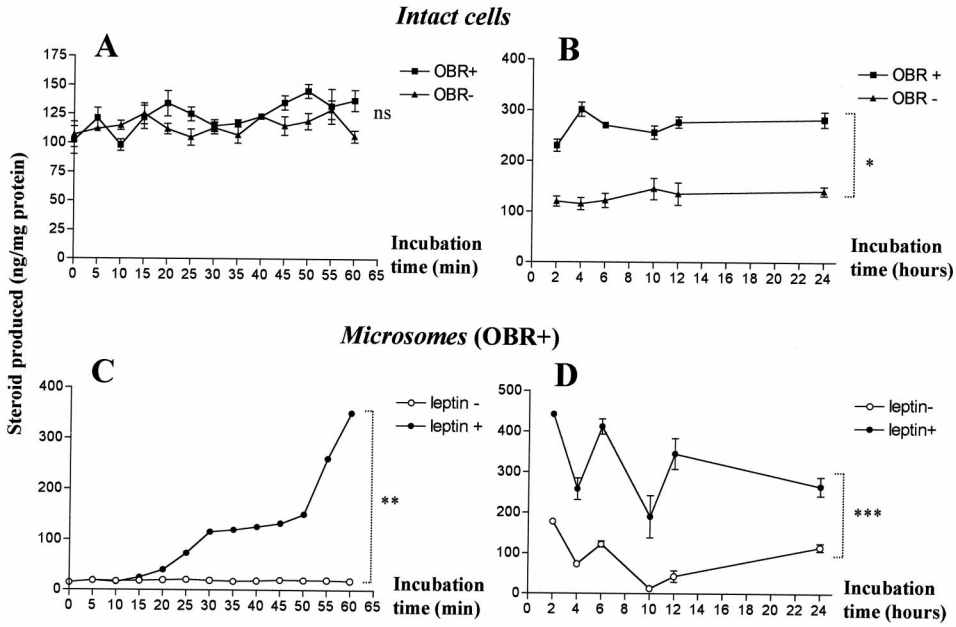

FIG. 3. Short- (A, C) and long-term (B, D) effect of leptin (30 pM) on CYP17 17a-hydroxylase activity in NCI-H295R in cells in *intact cells* (A and B) and in *microsomes* (OBR+) (C and D). OBR+ (■) were previously selected and separated from the OBR− cells (▲) by 6 or 24 h leptin treatment (30 pM). OBR+ cells were preincubated with 750 ng/ml steroidogenic precursors at time −6 h for the *intact cells* experiments and −30 min for the assay in *microsomes*, in the presence (●) or absence (○) of 30 pM leptin. In more details, 30 pM leptin were added at time 0 and incubations were discontinued at time 2, 4, 6, 10, 12, and 24 h, thereafter, when cell extracts were prepared, steroid extracted and assayed in duplicate. All values are expressed as mean ± SD and represent the result of three independent experiments. The data were subjected to *t* test analysis (paired): ns, nonsignificant; *, $P < 0.025$; ***, $P < 0.005$. Note the differences in *x*-axis scale.

17,20-lyase activity

Intact cells. That leptin stimulation is the result of a relatively fast phenomenon, is demonstrated by the short-term experiments: the DHEA content in NCI-H295R cells increased significantly already 15 min after leptin treatment initiation in OB-R + cells. Again, the OB-R negative cells did not respond to leptin treatment (Fig. 4A), suggesting an OB-R specific effect. In contrast to 17OH-progesterone, the DHEA synthesis increased progressively between 6 and 12 h after initiation of treatment, and stayed constant up to 24 h (Fig. 4B). This event appears to be OB-R dependent because the OB-R negative cells did not show any difference in CYP17 enzymatic activity upon leptin addition. *Microsomes* (OBR+): When the same experiment was carried out in the reconstituted system, no such differential effect was seen, suggesting that the cell-free system does contain the elements necessary to stimulate 17,20-lyase activity (Fig. 4C).

Because the abundance of the alternative redox partner cytochrome b_5 (CYB5) plays an important role in the regu-

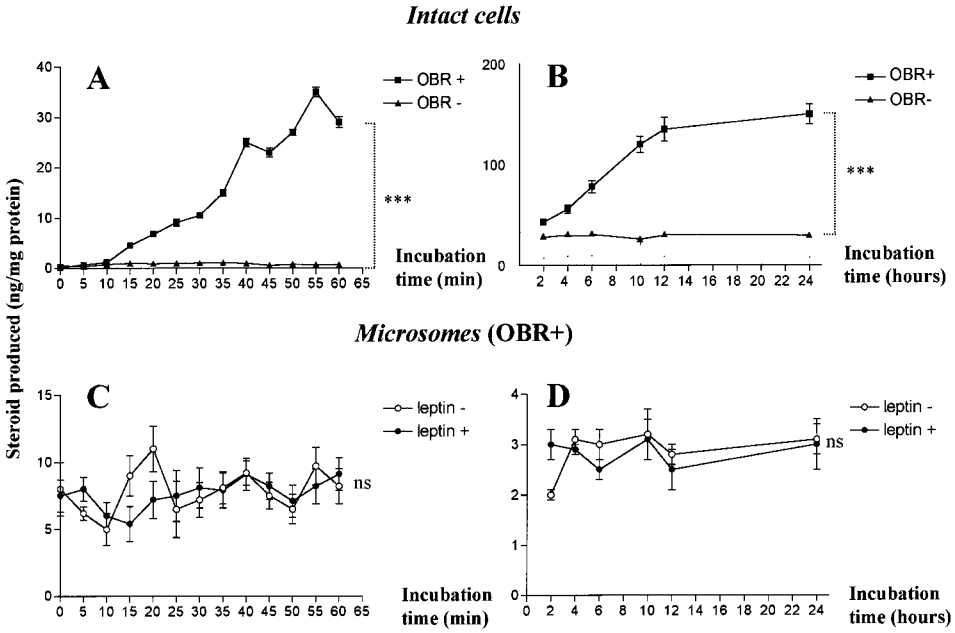

FIG. 4. Short (A, C) and long-term (B, D) effect of leptin (30 pM) on CYP17 17,20-lyase activity in NCI-H295R in cells in *intact cells* (A and B) and in reconstituted system (*microsomes,* OBR+) (C and D) (OBR+ ■; OBR− ▲). The experimental procedure and the symbols are the same as described for Fig. 3, with the exception of the time points: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. ns, Nonsignificant; **, $P < 0.01$; ***, $P < 0.001$. Note the difference in *x*-axis scale.

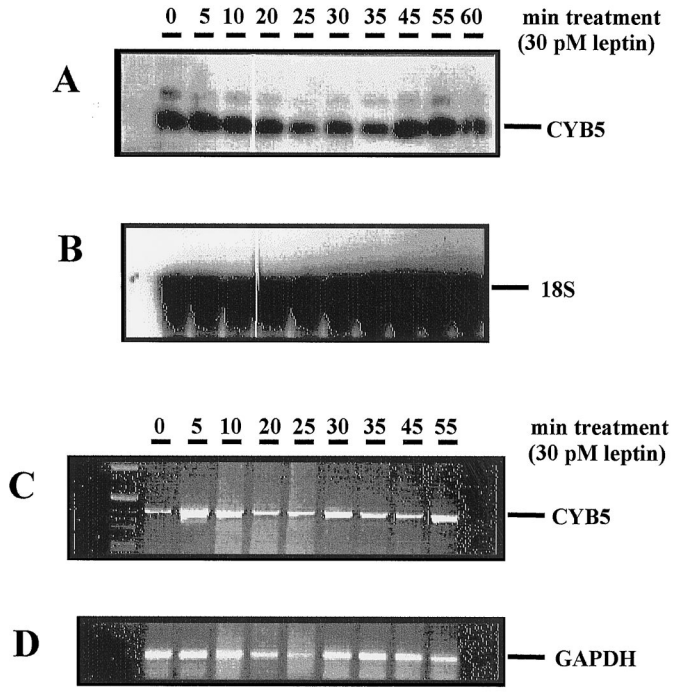

FIG. 5. CYB5 gene expression under short-term leptin treatment (30 pM), detected by Northern blot analysis (A), using 18S hybridization as loading control (B) and RT-PCR (C; D, GAPDH internal control) in OBR+ NCI-H295R cells. No correlation is detectable between the amount of *CYB5* message and 17,20-lyase activity.

lation of 17,20-lyase activity (12–14), we analyzed the expression of *CYB5* under short-term leptin treatment by Northern blot analysis and RT-PCR. As shown in Fig. 5, no positive correlation between CYB5 mRNA abundance and 17,20-lyase activity was detectable.

The phosphorylation state of CYP17 protein is significantly higher in the OB-R expressing cells ($P < 0.005$) but did not change upon leptin treatment (Fig. 6, A and C). The differences in phosphate incorporation are not due to variations in protein synthesis, as demonstrated by the comparable amount of 35S-methionine labeled CYP17 protein (Fig. 6B). The divergence in immunoprecipitable phosphorylated CYP17 protein between OBR+ and OBR− cells, remains significant when the amount of ^{32}P radioactivity is corrected by ^{35}S-dependent radioactivity. The same is true for the nonsignificant differences in phosphate incorporation under leptin treatment in both cell populations (Fig. 6C). This higher phosphate content in the CYP17 protein of leptin responsive cells correlates with 17,20-lyase activity, that appears to be higher in these cells already at the first treatment time points (Figs. 3 and 4). Although NIH-OvCar3 cells express CYP17, no phosphorylation of the protein was seen in these cells (not shown). The elimination of the O-phosphomonoesters from the hydroxyamino acids (ser/thr and tyr) by way of alkaline phosphatase treatment, selectively inhibited the DHEA formation in OBR expressing cells (Fig. 7A), without influence on the 17α-hydroxylase activity (Fig. 7B), confirming the dependence of 17,20-lyase activity on phosphate incorporation. The specificity of the effect was confirmed by the abolition of the differences in CYP17 activities upon inhibition of the phosphate removal by sodium-orthovanadate treatment (Fig. 7). The reliance of 17,20-lyase activity stimulation on posttranslational modification is confirmed by the fact that complete inhibition of novel protein synthesis (cycloheximide treatment, 8B), did not significantly alter the DHEA increase under leptin treatment in OBR+ cells (Fig. 8A) at early time points (2.5 h). The later decrease in DHEA production correlates with a reduction in CYP17 phosphorylated protein down to 25% of baseline (Fig. 8C) and not to decreased protein content, as demonstrated by the Western blot analysis (Fig. 8D). Whether this decline in phosphate incorporation depends on an influence of cycloheximide on the cellular phosphorylation/dephosphorylation equilibrium remains to be established. 17α-hydroxylase activity is

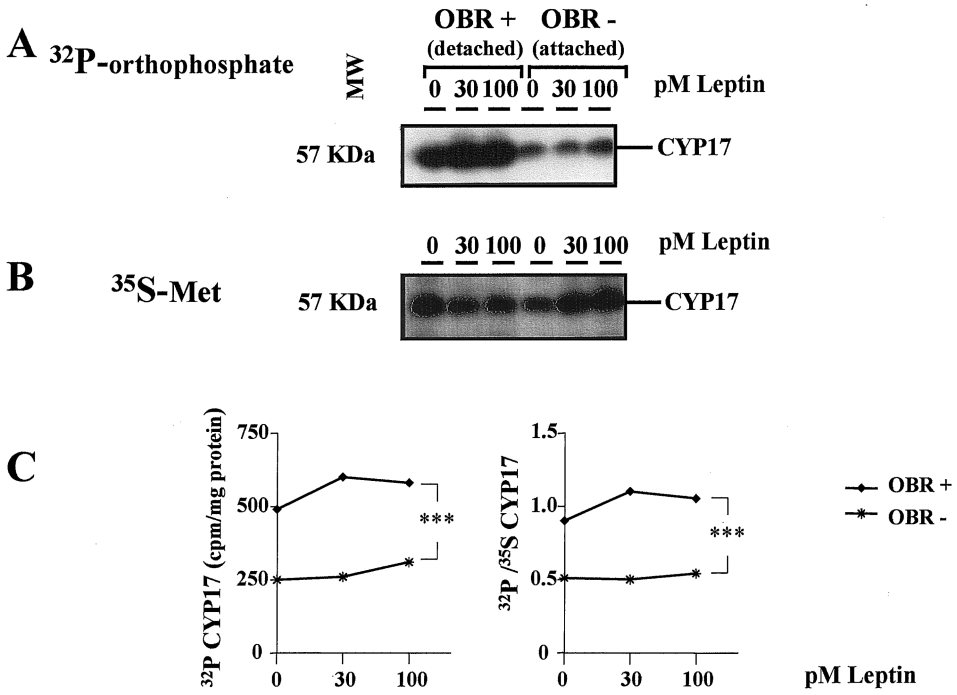

FIG. 6. Effect of leptin treatment on phosphorylation state of CYP17 protein in NCI H295R cells. MW, Molecular weight. A, Cells expressing OBR show a higher amount of immunoprecipitable phosphorylated CYP17 protein compared with the OBR− cells. B, The total amount of CYP17 protein did not change significantly under leptin treatment in OBR+ and OBR− cells, as demonstrated by the ^{35}S-methionine (^{35}S-Met) labeling. C, Quantification of radioactivity incorporation in the immunoprecipitated CYP17 protein. Although significant differences are present between cpm/mg protein between OBR+ and OBR− cells (***, $P < 0.005$), no significant difference in phosphate incorporation was detected in absence or presence of leptin within the same cell population. The data are representative of three independent experiments.

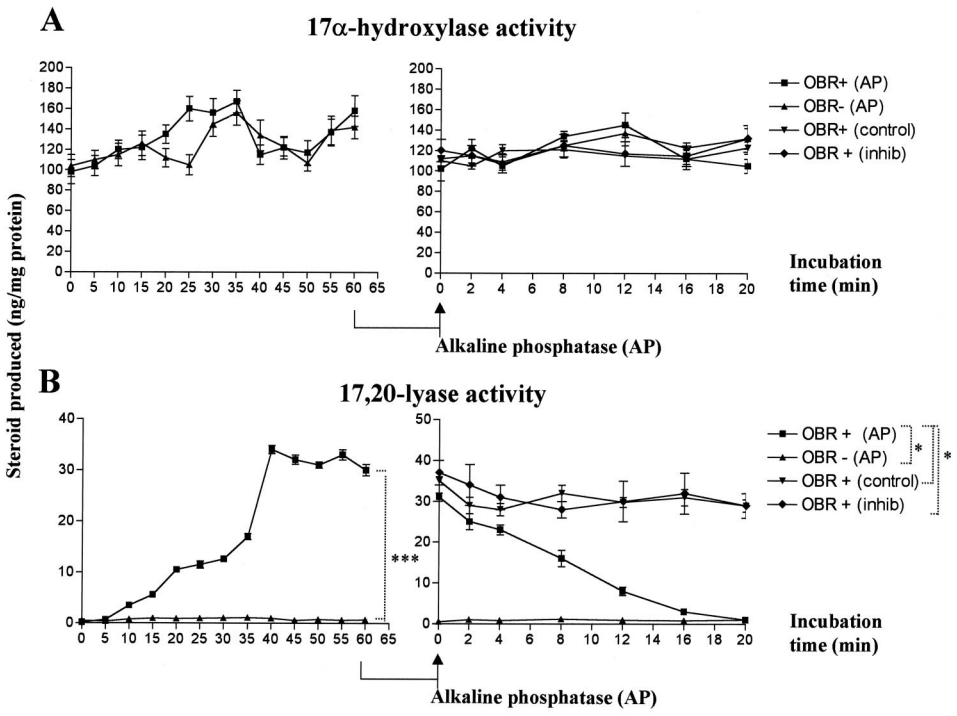

FIG. 7. Effect of dephosphorylation on the CYP17 enzymatic activities. During short-term treatment with 30 pM leptin, NCI-H295R cells (OBR+ ■; OBR− ▲) were analyzed for 17α-hydroxylase and 17,20-lyase activity in intact cells. The following phosphate removal (alkaline phosphatase) selectively abolishes the DHEA production in the reconstituted system (■). The effect is not an artifact of the experimental procedure because no change in DHEA production was seen when water was used as control (▼). The effect is specific because OBR− cells (▲) do not react to leptin treatment. As expected, dephosphorylation has no influence on 17α-hydroxylase activity. The addition of the phosphatase inhibitor sodium-orthovanadate (2 mM) to the lysis buffer eliminated the differences between the two activities in OBR + cells (♦). The data were subjected to *t* test analysis (paired): **, $P < 0.01$; ***, $P < 0.005$.

not surprisingly independent from phosphate incorporation (Fig. 8C).

To determine whether the observed increased in DHEA production in NCI-H295R cells in response to leptin is the result of a stimulation of either StAR, CYP11A1 (cholesterol side-chain cleavage) and/or an inhibition of 3β-hydroxysteroid dehydrogenase activity, unstimulated cells, and cells stimulated with 8Br-cAMP, leptin, or both were incubated in the presence of the hydroxylated cholesterol derivative 22R-HC. This compound, unlike cholesterol, readily diffuses to CYP11A1 to be converted to pregnenolone. The expression of StAR, CYP11A1, and ADX in NCI-H295R cells was demonstrated by RT-PCR (not shown). As shown in Fig. 9, the addition of leptin does not influence conversion of 22R-HC to progesterone in OBR + NCI-H295R cells alone or in combination with cAMP. The addition of cholesterol to the culture medium caused an inhibition of progesterone production in cAMP stimulated

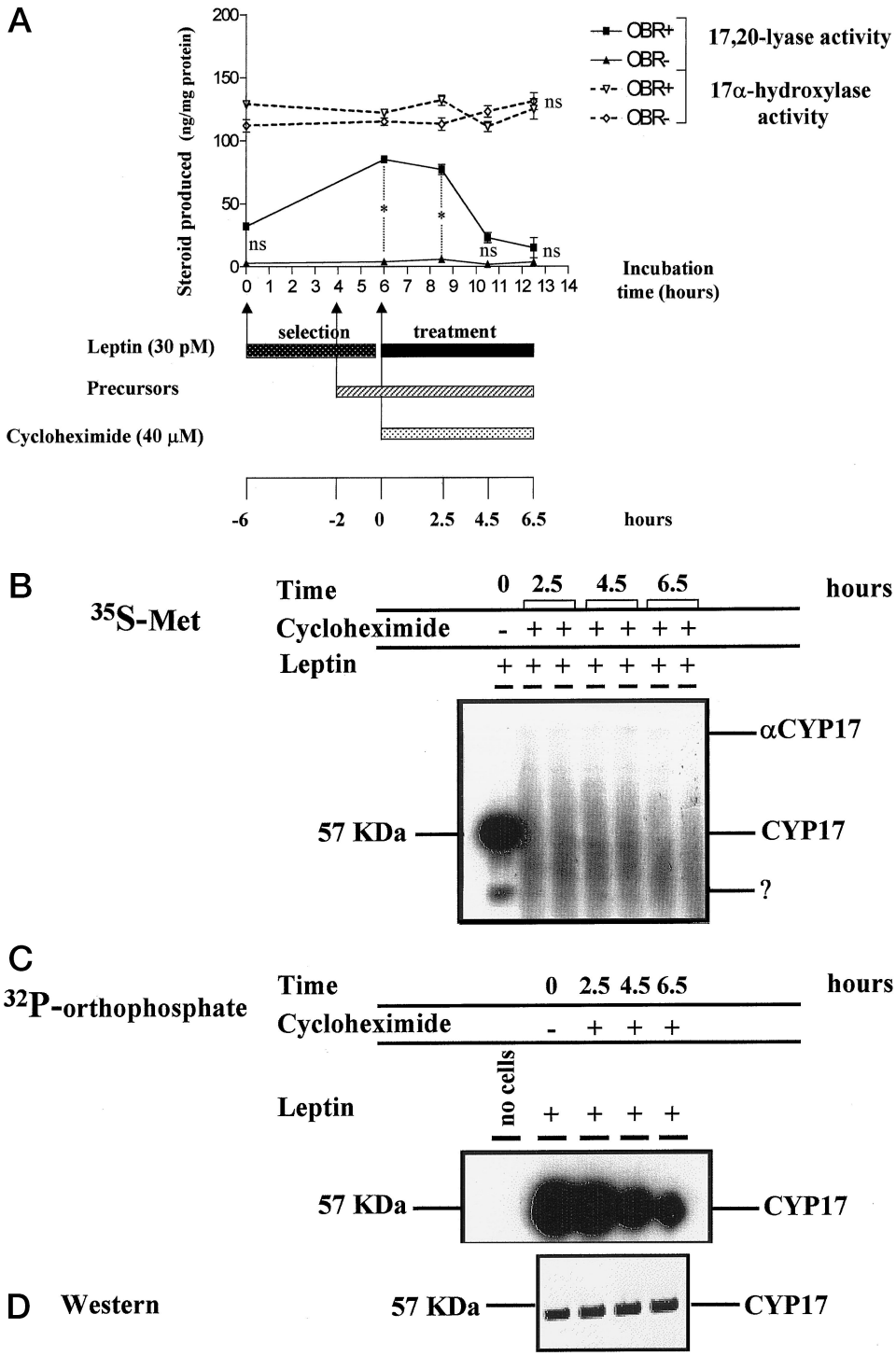

FIG. 8. Effect of protein synthesis inhibition on CYP17 enzymatic activities under leptin treatment. OBR + cells were selected with leptin treatment (6 h, *dotted dark block,* "selection") A, Leptin (*black block*, "treatment") and cycloheximide (*dotted light block*) treatment influence on CYP17 enzymatic activity in NCI-H295R cells. B, Cycloheximide suppresses novel protein synthesis already 2.5 h after initiation of treatment in OBR+ cells. C, Immunoprecipitable phosphorylated CYP17 protein in leptin/cycloheximide treated NCI-H295R cells. 17,20-lyase activity is not significantly reduced until phosphorylated CYP17 protein is decreased to 25% of baseline (*close symbols, solid line*). The changes are not due to diminished CYP17 content because no remarkable differences in the Western blot analysis were seen (D). 17α-hydroxylase activity remains unchanged at all time points (*open symbols, broken lines*). The data are expressed as mean ± SD and represent the result of three independent experiments. The data have been subjected to *t* test analysis (paired). ns, Nonsignificant; *, $P < 0.025$.

cells, but not in basal conditions. At present time, we have no explanation for this phenomenon.

Discussion

CYP17 is the key for the regulation of the biosynthetic route of pregnenolone to its various final products. Regulation is only possible through the differential control of the two distinct enzymatic activities incorporated in this protein: 17α-hydroxylase and 17,20-lyase. The dual function of this enzyme allows direction of the steroid precursors along several pathways: 1) 17α-hydroxylated products with the intact side chain are precursors of cortisol, as in adrenal *zona fasciculata*; 2) generation of C19 steroids by both, 17α-hydroxylation and 17,20-cleavage, directs substrates toward the formation of sex hormones, as in adrenal *zona reticularis* and in the gonads; 3) in the adrenal *zona glomerulosa,* which

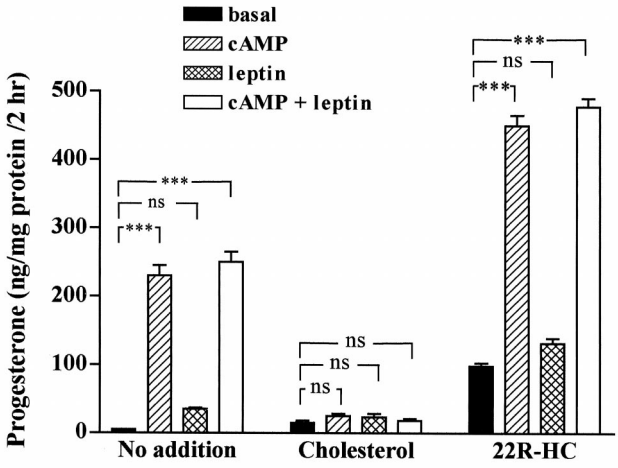

FIG. 9. Effect of leptin treatment on progesterone production. NCI-H295R cells (expressing OBR) were maintained in control conditions (no addition) or treated with 50 μM cholesterol or 22R-hydroxy cholesterol in the absence (basal, *black bars*) or presence of 200 μM 8Br-cAMP (cAMP, *hatched bars*) for 30′ with (*white bars*) or without (*checked bars*) preincubation with 30 pM leptin for 6 h. The data are expressed as mean ± SD of three independent experiments, and subjected to *t* test analysis (paired); ns, nonsignificant; ***, $P < 0.005$. Although the system can be stimulated by cAMP, leptin appears to have no influence on progesterone production, suggesting no direct effect of leptin on either StAR, CYP11A1, or 3β-hydroxysteroid dehydrogenase activities.

lacks the CYP17 activities, pregnenolone is converted to mineralocorticoids.

The two activities of CYP17 are differentially regulated in a tissue- and time-dependent fashion. The redox partner abundance and posttranslation modifications, such as phosphorylation, have been demonstrated to play an essential role in the activation of 17,20-lyase activity (10–14). Nevertheless, the physiological factor(s) involved in such activation are still unknown. Clarification of the control mechanisms governing the two activities is an essential step in the understanding of adrenarche and the onset of puberty.

Among the several possible agents, leptin has recently gained importance. It acts through the leptin receptor OB-R, a single-transmembrane-domain receptor of the class I cytokine receptor family (15). These receptors are known to act through JAK and STAT proteins. Typically, JAK proteins are constitutively associated with membrane-proximal sequences of the receptor intracellular domain (ICD) and phosphorylate tyrosine residues of the receptor ICD upon ligand binding. The phosphorylated ICD then provides a binding site for a STAT protein, which is then activated. The activated STAT protein then translocates to the nucleus and stimulates transcription. In the case of OB-R, JAK 2 and STAT3 are involved in the signal transduction and transcriptional activation. Because the OB-R signal transduction pathway operates through phosphorylation of JAK and STAT proteins, the mechanism underlying the observed events most likely involves phosphorylation, a process already shown to be crucial for 17,20-lyase activity (10).

Leptin can exert its action on multiple targets. In fact, although it appears that posttranslation modifications are crucial for 17,20-lyase activity, the role of substrate accessibility in the increased DHEA production must be assessed. Because the precursor for all steroids is cholesterol, leptin might influence cholesterol availability by affecting either its transfer to the inner mitochondrial membrane or the rate-limiting step of steroidogenesis, *i.e.* the cleavage of the side chain of cholesterol to yield pregnenolone. Possible targets of such action would therefore be steroidogenic acute regulatory protein (StAR) or cytochrome P450 side chain cleavage (CYP11A1), respectively. The mechanism regulating this putative influence likely relies on phosphorylation. In fact, although phosphorylation might not be required for StAR mitochondrial import, phosphorylation of StAR is directly linked to steroidogenic response of the cell to hormone stimulation. (18). The fact that the enzymatic system is below saturation conditions, grants the bases for this hypothesis. We therefore performed experiments to assess the possibility that the rise in DHEA production under leptin treatment is due to stimulation of StAR and/or CYP11A1 activities, or inhibition of conversion of DHEA to androstenedione, catalyzed by 3β-hydroxysteroid dehydrogenase. The lack of influence of leptin on progesterone production suggests that this is not the case, and reinforces the thesis of a direct effect of leptin on CYP17 catalytic potential.

Another possible mechanism by which leptin stimulates 17,20-lyase activity may reside in an increase of the availability of cytochrome b_5, the alternative electron donor for CYP17. The absence of a positive correlation between *CYB5* gene expression and 17,20-lyase activity implies that leptin-stimulated DHEA production is not primarily attributable to an increase of b_5:CYP17 ratio.

The effect of leptin on 17α-hydroxylase activity seems to be consistently independent from cell integrity, suggesting that the underlying mechanisms are different from the OBR second messenger pathway. Nevertheless, this effect is dependent on the presence of leptin and its receptor. Further studies are necessary to clarify whether the observed phenomenon represents a specific event or an experimental artifact.

On the other hand, our data suggest for the first time a direct role of leptin already at physiological concentrations in the regulation of 17,20-lyase activity. This influence is direct, OB-R specific and depends on the integrity of the signal transduction pathway. The effect of leptin treatment can be seen at early stages, implicating a role of leptin in the acute regulation of adrenal androgen synthesis, probably associated with posttranslational modifications. The dependence of 17,20-lyase activity on the phosphorylation state is confirmed by two lines of evidence: first, the differences in phosphorylation state of CYP17 in OBR+ and OBR− cells correlate to the differences in DHEA production in these two cell populations. The precise mechanism responsible for these observation remains to be elucidated. Second, the loss of 17,20-lyase activity after O-phosphomonoesters removal by alkaline phosphatase. The fact that inhibition of the dephosphorylation (sodium-orthovanadate treatment) abolishes the phenomenon further corroborates the hypothesis. Moreover, the effect of leptin treatment on 17,20-lyase activity is not dependent upon new protein synthesis because complete block of translation does not change the increment in DHEA production. Such DHEA synthesis, therefore, ap-

pears to rely on the presence of phosphorylated CYP17 protein. The lack of differences in immunoprecipitable phosphorylated CYP17 protein under leptin treatment, suggests the need of more specific assays for the detection of single phosphorylated residues in the CYP17 protein. Nevertheless, a correlation between the basal phosphorylation state of CYP17 protein and basal 17,20-lyase enzymatic activity appear to exist, as demonstrated by the differences in DHEA production between OBR+ and OBR− NCI-H295R cells. It remains to be established whether phosphorylation must take place directly on CYP17 or engages some other intermediary protein that, once activated, in turn, promotes phosphorylation of CYP17.

Our results contrast with those of others (16) reporting a leptin-dependent down-regulation of CYP17 mRNA with consequent decrease of steroid production in human adrenal cells. Several points can be made to explain this discrepancy. First, the leptin doses used in our experiment, resembling closer the physiological concentrations, are much lower than those used by Glasgow *et al.* Second, incubation time appears to play a role, at least with regard to the 17α-hydroxylase activity. Third, it appears that the reported inhibitory effect of leptin is not direct because no data are presented showing the influence of leptin alone, *i.e.* without ACTH on CYP17 mRNA or activity. Although ACTH is essential for the regulation of cortisol production in the *zona fasciculata*, it appears to play a more marginal role in governing the 17,20-lyase activity in the *zona reticularis*, as demonstrated by the lack of increase of ACTH levels at adrenarche (17). Furthermore, the described cases of either leptin deficiency (3) or leptin resistance (4) show no abnormal ACTH or cortisol levels, suggesting the absence of gross defects in the hypothalamic-pituitary adrenal axis and implicate a more marginal role of leptin in the physiological regulation of 17α-hydroxylase activity. On the other hand, the lack of production of any sex hormones, mirrored by the failure of sexual development in these patients, confirms the physiological importance of leptin in the stimulation of the 17,20-lyase activity of CYP17.

In conclusion, we demonstrated that leptin in physiological amounts is important in the differential regulation of 17,20-lyase *vs.* 17α-hydroxylase activity of CYP17 enzyme. The action of leptin appears to imply the control of CYP17 phosphorylation, it requires the presence of the leptin receptor and the integrity of the OB-R signal transduction pathway, without the need of concomitant activation of other signal transduction routes, such as that coupled to the MC2-receptor (ACTH receptor). Our data implicate leptin in the acute, although not immediate, and long-term stimulation of 17,20-lyase activity in human adrenal cells and represent a step forward in the understanding of the mechanisms governing adrenarche.

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