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

ANNA BIASON-LAUBER, MILO ZACHMANN, AND EUGEN J. SCHOELEN

Department of Pediatrics, University of Zurich, Division of Endocrinology/Diabetology and Clinical Chemistry/Biochemistry, 8032 Zurich, Switzerland

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 OB-R signal transduction pathway, and a process already shown to be crucial for 17,20-lyase activity. (Endocrinology 141: 1446–1454, 2000)

ADRENAлёHE 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 developmental stages (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 were 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 vendor. For the initial plating, 7.5 × 10⁴ cells were seeded on 35-mm plates. Medium was replaced every 24 h. Experiments were performed
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In vivo metabolic labeling and immunoprecipitation

Cells were metabolically labeled with either \(^{32}P\) orthophosphate (\(^{32}P\)Pi) (200 \(\muCi/ml\), NEN Life Science Products, Boston, MA) for 1 h in phosphate-free medium (Life Technologies, Inc. 11963–022) or with \(^{35}S\)-methionine (100 \(\muCi/ml\), NEN Life Science Products, easytag) for 2 h in methionine-free medium (Life Technologies, Inc.). Labeled cells were lysed in 1 

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5 M MgCl\(_2\), 1 mM EDTA, 1% Triton-X, 10% glycerol in the presence of protease inhibitors (phenylmethylsulfonyl fluoride 34 \(\mug/ml\), 0.7 \(\mug/ml\) pepstatin, 5 \(\mug/ml\) leupeptin, Roche Molecular Biochemicals) and phosphatase inhibitors for the phosphorylation experiments (100 mM sodium-fluoride, 10 mM sodium-pyrophosphosphate, 2 mM sodium-orthovanadate, Sigma). The lysates were clarified by centrifugation at 15,000 \(\times g\) 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 \(^{35}P\) 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

\textit{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 \(\times\) 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.

\textit{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 \(\mug\) of microsomal protein with 500 nmol of progesterone or 17OH-pregnenolone for 30 min, as previously described (10).

\textit{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.

\textit{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 \(\mug\) 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 \(\mul\) RIA buffer (20 mm K\(_2\)HPO\(_4\), 47 mm Na\(_2\)HPO\(_4\), 2H\(_2\)O, 5 mm NaN\(_3\), 1 g/liter bovine y-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 \(\pm\) so 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). Reploting 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 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.

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 OB-R 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 OB-R+ and OB-R- cells: 17OH-progesterone goes from 100 ± 5 to 403 ± 10 in OB-R+ and from 110 ± 10 to 390 ± 7 ng/mg protein in OB-R- cells; DHEA in OB-R+ cells: basal 75 ± 4 ng/mg protein, stimulated 350 ± 7 ng/mg protein; in OB-R- 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 (OB-R+):** 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 OB-R+ 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).
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

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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 (OB-R+): 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 b5 (CYB5) plays an important role in the regul-
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 OB-R+ and OB-R− cells, remains significant when the amount of 32P radioactivity is corrected by 35S-dependent radioactivity. The same is true for the non-significant 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 OB-R 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 OB-R+ 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. 8A) 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
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 8β-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...
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...
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 posttranslational 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 posttranslational modifications are crucial for 17,20-lyase activity, the role of substrate accessi-

**Fig. 9.** Effect of leptin treatment on progesterone production. NCI-

- **basal**
- **cAMP**
- **leptin**
- **cAMP + leptin**

<table>
<thead>
<tr>
<th>Progesterone (mg/mg protein/2 hr)</th>
<th>No addition</th>
<th>Cholesterol</th>
<th>22R-HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>basal</strong></td>
<td>500</td>
<td>400</td>
<td>300</td>
</tr>
<tr>
<td><strong>cAMP</strong></td>
<td>450</td>
<td>350</td>
<td>250</td>
</tr>
<tr>
<td><strong>leptin</strong></td>
<td>400</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td><strong>cAMP + leptin</strong></td>
<td>450</td>
<td>350</td>
<td>200</td>
</tr>
</tbody>
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![Graph](https://academic.oup.com/endo/article-abstract/141/4/1446/2988164)
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 for 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 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 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 implicating 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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