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Decreased Expression of Steroidogenic Acute Regulatory Protein: A Novel Mechanism Participating in the Leptin-Induced Inhibition of Glucocorticoid Biosynthesis

NADIA CHERRADI, ALESSANDRO M. CAPPONI, ROLF C. GAILLARD, AND FRANÇOIS P. PRALONG

Division of Endocrinology, Diabetology and Metabolism, University of Lausanne Medical School (R.C.G., F.P.P.), CH-1011 Lausanne, Switzerland; and Division of Endocrinology and Diabetology, University of Geneva Medical School (N.C., A.M.C.), CH-1211 Geneva 14, Switzerland

The adipocyte-derived hormone leptin is a central modulator of food intake, metabolism and neuroendocrine functions. It is also involved in a physiological loop linking the activity of the hypothalamo-pituitary-adrenal axis and adipose tissue. At the adrenal level, leptin has been shown to antagonize the effects of ACTH on glucocorticoid biosynthesis by decreasing the expression of various enzymes of the steroid biosynthetic pathway. The steroidogenic acute regulatory protein regulates cholesterol delivery to the $P450_{scc}$ enzyme, a process that is rate limiting in steroid hormone biosynthesis. We have demonstrated here that leptin significantly inhibits the expression of steroidogenic acute regulatory protein in primary cultures of rat adrenocortical cells. This inhibition was observed at both the protein and mRNA levels. In contrast, leptin was not found to interfere with the expression of the cytosolic enzyme cholesterol ester hydrolase or with that of the mitochondrial enzyme $P450_{scc}$. In addition, we observed the anticipated stimulation of cAMP production by ACTH in the presence of leptin, suggesting that it does not interfere with intracellular ACTH signaling. In summary, our data provide evidence that the interplay existing between leptin and ACTH *in vivo* is mediated at least partially via a direct and opposite modulation of steroidogenic acute regulatory protein, a key factor in the adrenal steroid biosynthetic pathway. This effect of leptin could also be relevant to other steroidogenic tissues. (*Endocrinology* 142: 3302–3308, 2001)

THE MAIN FUNCTION of leptin, the product of the *ob* gene (1), is probably to signal body fat stores to the satiety centers of the hypothalamus (2–4). It is also involved in various endocrine regulations (5). Although most of the effects of leptin were initially thought to occur at the central level, several reports have now demonstrated the expression of the biologically active isoform of the leptin receptor in the endocrine pancreas (6), the ovary (7), or the placenta (8). Consistent with this widespread expression, leptin can directly modulate the activity of these glands; it has been shown to inhibit insulin secretion from pancreatic β-cells (9), it decreases the production of estradiol and progesterone from ovarian granulosa cells (10, 11), at least partly via inhibition of the electron transport protein adrenodoxin (11), and it can modulate the release of hCG from human trophoblast cells in culture (12).

In recent years, leptin has emerged as an important physiological regulator of the hypothalamo-pituitary-adrenal (HPA) axis (13–16). In this setting, a direct modulation of glucocorticoid biosynthesis and secretion by leptin has been described (17, 18). As glucocorticoids are known to stimulate leptin secretion (19, 20), adipose tissue and the corticotrope axis seem to be interconnected in a classical endocrine loop (21–23).

The cellular and molecular mechanisms underlying the adrenal effects of leptin have been only partially elucidated. A leptin-induced inhibition of the expression of the steroidogenic enzymes cytochrome P450 C21-hydroxylase ($P450_{C21}$), side-chain cleavage ($P450_{SCC}$) and C17α-hydroxylase ($P450_{17\alpha}$) in the bovine adrenal gland has been described (18, 24). However, other intracellular targets of leptin's effects have been suggested in the rat (17). In view of the potential pathophysiological importance of this peripheral effect of leptin, we investigated its capacity to modulate the early steps of steroidogenesis by interfering with expression of the steroidogenic acute regulatory protein (StAR).

StAR is a key element in the rate-limiting step of steroid hormone biosynthesis; it regulates cholesterol delivery to the $P450_{SCC}$ enzyme located in the inner mitochondrial membrane (25, 26). Mutations in the StAR gene have been shown to underlie lipoid congenital adrenal hyperplasia, a disorder leading to a dramatic congenital deficiency in all steroid hormones (27, 28). Recent evidence suggests that StAR can act as a sterol transfer protein (29). However, its mechanism of action on cholesterol transfer remains to be elucidated.

Using primary cultures of rat adrenocortical cells, we were able to demonstrate that leptin inhibits the adrenal expression of StAR. This effect was apparent at both the mRNA and protein levels, thus providing a novel mechanism of action for leptin in modulating the function of steroidogenic tissues.

Materials and Methods

Chemicals

Leptin was obtained from PeproTech EC Ltd. (London, UK), and ACTH was obtained from Sigma (Buchs, Switzerland). [^{3}H]Pregnenolone was purchased from Amersham Pharmacia Biotech (Zurich, Switzerland). Antipregnenolone antiserum was obtained from Biogen-

Abbreviations: CEH, Cholesterol ester hydrolase; HPA, hypothalamo-pituitary-adrenal; StAR, steroidogenic acute regulatory protein.

esis (Poole, UK). Antisera against the $P450_{scc}$ and the cholesterol ester hydrolase (CEH) enzymes were provided by Dr. G. Defaye (Commissariat à l'Energie Atomique, Grenoble, France) and F. B. Kraemer (Stanford, CA), respectively. Win 19758 was purchased from Farillon (London, UK). Hybond-N^+ membranes, Rapid Hybridization Buffer, and Rediprime random primer labeling kit were supplied by Amersham Pharmacia Biotech. All other chemicals used were purchased from Sigma (St. Louis, MO) or Fluka (Buchs, Switzerland).

Rat adrenal cell culture

Before death, animals were housed in our animal facility under a 12-h light, 12-h dark schedule and fed *ad libitum*. All animal care and scientific procedures were carried out in strict accordance with our government directives and after formal approval by the State Veterinary Department.

Wistar female rats, weighing 200–250 g, were killed by decapitation, adrenals were rapidly removed, and the medulla was separated from the cortex by squeezing the gland gently after making an incision through the capsula. Dispersion of adrenal glands was performed as described previously (17). Briefly, adrenals were minced with a scalpel blade and then subjected to combined enzymatic and mechanical dispersion; tissue fragments were placed in a Bellco flask (Vineland, NJ) to allow constant trituration and were incubated for 90 min at 37 C in the presence of collagenase type I (Sigma), followed by neuraminidase type V (Sigma). After dispersion, cells were resuspended in medium containing 2.5% FCS and plated at a concentration of 250,000 cells/well in 24-well multiwell plates pretreated with poly-D-lysine. They were incubated for 72 h at 37 C in 95% air/5% CO_2. Medium was then changed for serum-free medium, and stimulations were performed as described below. Viability was assessed by trypan blue exclusion and was always more than 90%.

Experimental design

To investigate the long-term effects of leptin (*i.e.* possibly at the level of gene expression) on the acute stimulation of glucocorticoid biosynthesis, cells were preincubated for 24 h in the absence or presence of murine recombinant leptin (10^{-7} M) in serum-free medium. After this preincubation, medium was changed, and cells were stimulated acutely for 90 min with ACTH (10^{-9} M) in the presence of the same leptin concentration as during the preincubation period. At the end of the ACTH stimulation, medium was collected and immediately frozen until assay for pregnenolone, and cells were lysed for protein or mRNA extraction.

In parallel sets of experiments, cells were stimulated for 24 h with ACTH (10^{-9} M), leptin (10^{-7} M), or the combination of ACTH (10^{-9} M) and leptin (10^{-7} M).

Steroid and cAMP measurements

For the assessment of pregnenolone production, WIN 19758 (5 μM) was included in the incubation medium to prevent conversion of pregnenolone into progesterone. At the end of the incubation period, pregnenolone was determined directly in the medium by RIA. Steroid production was normalized and expressed per mg cellular protein. cAMP levels were determined using a commercially available kit supplied by Amersham Pharmacia Biotech. Assay was performed directly in culture medium, according to the instructions of the manufacturer.

SDS-PAGE

SDS-PAGE was performed according to the Laemmli method (30). At the end of the stimulation period, cells were washed three times with ice-cold PBS and lysed in 10 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 1% Triton, 1% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The lysate was cleared by centrifugation for 10 min at 12,000 $\times$ *g* at 4 C. Total cell protein extracts (10 μg/lane) were solubilized in sample buffer [60 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue] and loaded onto a 12% SDS-PAGE minigel (Mini Protean II System, Bio-Rad Laboratories, Inc., Richmond, CA). Electrophoresis was performed at 150 V for 1 h.

Blotting procedure and immunodetection

SDS-PAGE-resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Switzerland) according to Towbin *et al.* (31). After transfer, the membrane was incubated in a blocking buffer (PBS buffer containing 0.4% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature, and then further incubated either with an antiserum generated by CovalAb (Oullins, France) against a peptide fragment (amino acids 88–98) of StAR (1 h in PBS/Tween buffer) or with antiserum specific for the P450scc (2 h in PBS/Tween buffer) or for the cholesterol ester hydrolase enzyme (12 h in PBS/Tween 20 buffer). The membrane was thoroughly washed with the same buffer (three times, 10 min each time), then incubated for 1 h with horseradish peroxidase-labeled goat antirabbit IgG (CovalAb). The nitrocellulose sheet was then washed four times for 15 min each time, and the antigen-antibody complex was revealed by enhanced chemiluminescence, using the Western blotting detection kit and HyperECL film from Amersham Pharmacia Biotech.

RNA isolation and Northern blot analysis

Adrenocortical cell total RNA was extracted using Tripure Isolation Reagent from Roche Molecular Biochemicals (Mannheim, Germany) according to the instructions of the manufacturer. For Northern blot analysis, 15–20 μg RNA were size-fractionated on a 1% formaldehyde agarose gel, vacuum-transferred onto Hybond-N^+ membranes, and fixed by UV cross-linking. The integrity of the 18S and 28S RNA was checked by ethidium bromide staining of the gel. Hybridization was performed using the previously cloned 1.5-kb mouse StAR cDNA (25). The cDNA was labeled with α-^{32}P using the Rediprime random primer labeling kit. Northern blots were prehybridized in Rapid Hybridization Buffer at 65 C for 30 min. The α-^{32}P-labeled probe (SA, 2×10^6 cpm/ng DNA) was then added, and the incubation was continued for 2 h at 65 C. Blots were washed for 5 and 15 min successively at room temperature in 2 $\times$ SSC (standard saline citrate)/0.1% SDS, then for 15 min in 1 $\times$ SSC/0.1% SDS. The final wash was performed at 65 C for 15 min in 1 $\times$ SSC/0.1% SDS. RNA-cDNA hybrids were visualized on Hyperfilms after a 12- to 24-h exposure period. Blots were stripped and reprobed with mouse GAPDH cDNA (Ambion, Inc., Lugano, Switzerland) to assess RNA loading.

Data analysis

Results are expressed as the mean $\pm$ SEM of three separate experiments. Each condition was performed in triplicate in each separate experiment. The mean values were compared by ANOVA using Fisher's test. $P < 0.05$ was considered statistically significant. Quantification of immunoblots and autoradiograms was performed using a computing densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Leptin inhibits ACTH-induced pregnenolone biosynthesis

To demonstrate the existence of the previously unrecognized intracellular target(s) of leptin within the steroidogenic cascade, we investigated the effects of leptin on the production of pregnenolone, the first steroid formed after cholesterol side-chain cleavage by the cytochrome $P450_{scc}$. As observed for corticosterone (17), leptin alone did not affect pregnenolone production. Furthermore, leptin added concomitantly with ACTH had no effect on pregnenolone production stimulated by 24 h of exposure to ACTH (data not shown). Figure 1 shows the steroidogenic response of adrenocortical cells exposed to leptin for 24 h and then stimulated for 90 min with ACTH. Values are expressed as a percentage of basal pregnenolone secretion (control cells). ACTH induced a substantial increase in pregnenolone production (305 $\pm$ 10% of controls; n = 3; $P < 0.001$). In leptin-pretreated cells, the acute response to ACTH was significantly altered

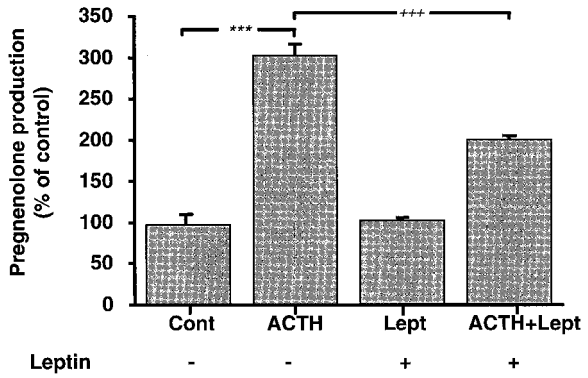

FIG. 1. Leptin inhibits ACTH-stimulated pregnenolone synthesis. Adrenocortical cells were preincubated for 24 h in the absence (−) or presence (+) of leptin (10^{-7} M). Incubation media were then changed, and cells were further stimulated with ACTH (10^{-9} M), leptin (10^{-7} M), or both for 90 min. Each value is the mean ± SEM of triplicate determinations from three independent experiments. ***, Significantly different from control (Cont), $P < 0.001$; 2++, significantly different from ACTH, $P < 0.001$.

(203 ± 2% of controls; n = 3; $P < 0.001$ *vs.* ACTH alone). Leptin by itself had no effect on pregnenolone secretion (105 ± 2% of controls; n = 3). These results indicated that a potential target for the inhibitory mechanism of leptin resides upstream of the production of pregnenolone.

Neither cAMP formation, nor CEH or P450$_{scc}$ expression is affected by leptin

In theory, various potential targets for the inhibitory action of leptin could exist along the early steps of the steroidogenic cascade triggered by ACTH in adrenocortical cells. Table 1 shows that the expected production of cAMP upon ACTH stimulation was identical in the absence and presence of leptin, thus demonstrating that leptin did not impede upon the generation of this intracellular signal.

Alternatively, leptin could affect the free cholesterol supply to the mitochondria or intramitochondrial cholesterol transfer to the P450$_{scc}$. Free cholesterol is generated through the action of CEH on cholesterol esters within intracellular lipid droplets. We therefore examined the effects of leptin on CEH and P450$_{scc}$ proteins expression (Fig. 2). Immunoblotting analysis of total cell extracts revealed that neither CEH nor P450$_{scc}$ protein content was affected by short-term stimulation with ACTH. This observation is in agreement with previous data showing that the induction of steroidogenic enzyme expression requires long-term exposure to trophic hormones (32). Similarly, no significant changes in both enzyme levels were observed in leptin-pretreated cells when exposed to ACTH. Finally, leptin alone had no effect on CEH or P450$_{scc}$ expression.

Together, these results suggested that the target of the inhibitory mechanism of leptin on ACTH-induced pregnenolone synthesis resides downstream of the production of the cAMP signal and cholesterol ester hydrolysis. As the expression of the P450$_{scc}$ enzyme was not affected by leptin, we examined the effect of leptin on the expression of StAR, the key protein in cholesterol transfer to the P450$_{scc}$.

TABLE 1. Leptin does not affect ACTH-induced cAMP levels in adrenocortical cells

24 h	90 min	cAMP (fmol/ml)
Control	Control	29.8 ± 6.6
	ACTH (10^{-9} M)	56043 ± 10609[a]
Leptin (10^{-7} M)	Control	17.8 ± 1.3
	ACTH (10^{-9} M)	55157 ± 9112[a]

Rat adrenocortical cells were preincubated for 24 h in the absence or presence of leptin. Incubation media were then changed, and cells were further stimulated with ACTH, leptin, or both for 90 min. Each value is the mean ± SEM of triplicate determinations from four independent experiments.

[a] Significantly different from control, $P < 0.001$.

Leptin inhibits ACTH-induced increase in StAR protein expression

Trophic hormone-activated steroidogenesis involves an increased transfer of cholesterol from the outer to the inner mitochondrial membrane (33), accompanied by an increase in StAR protein expression and accumulation within mitochondria (26, 34). To determine whether leptin affected StAR protein expression induced by ACTH, total protein extracts from adrenocortical cells were analyzed by immunoblotting. Figure 3A (*right panel*) shows StAR protein expression in adrenocortical cells preincubated for 24 h in the absence or presence of leptin, then exposed for 90 min to ACTH, leptin, or both agents. ACTH induced an increase in StAR protein content to 156 ± 8% of the control value (n = 3; $P < 0.01$). Pretreatment with leptin almost completely prevented the ACTH-induced increase in StAR protein (113 ± 7% of controls; $P < 0.05$ *vs.* ACTH). Leptin alone had no significant effect (107 ± 16% of controls; n = 3).

Leptin inhibits the ACTH-induced increase in StAR mRNA levels

The above results prompted us to examine whether leptin could exert its antisteroidogenic action by acting directly on StAR mRNA production in adrenocortical cells. Two different transcripts, one of 1.6 kb and the other of 3.4 kb, hybridized with StAR cDNA in Northern blot analysis (Fig. 3B, *left panel*). As they both showed a coordinate regulation, only the most abundant 3.4-kb product was quantified. The acute stimulation of adrenocortical cells with ACTH led to an increase in StAR mRNA to 265 ± 35% of controls (n = 3; $P < 0.01$; Fig. 3B, *right panel*). Pretreatment with leptin markedly reduced ACTH-elicited up-regulation of StAR transcripts to 160 ± 43% of controls (n = 3; $P < 0.05$ *vs.* ACTH; Fig. 3B, *right panel*). Leptin alone had no significant effect (107 ± 4% of controls; n = 3).

Discussion

Besides well recognized effects on the central control of food intake and energy metabolism (35), leptin has a prominent role in modulating the activity of the HPA axis (13). In mice the stimulation of corticosterone secretion induced by starvation or restraint stress can be partially counteracted by concomitant administration of leptin (5, 15). In the human the observation that circulating levels of leptin and cortisol are inversely related provides another indication that leptin

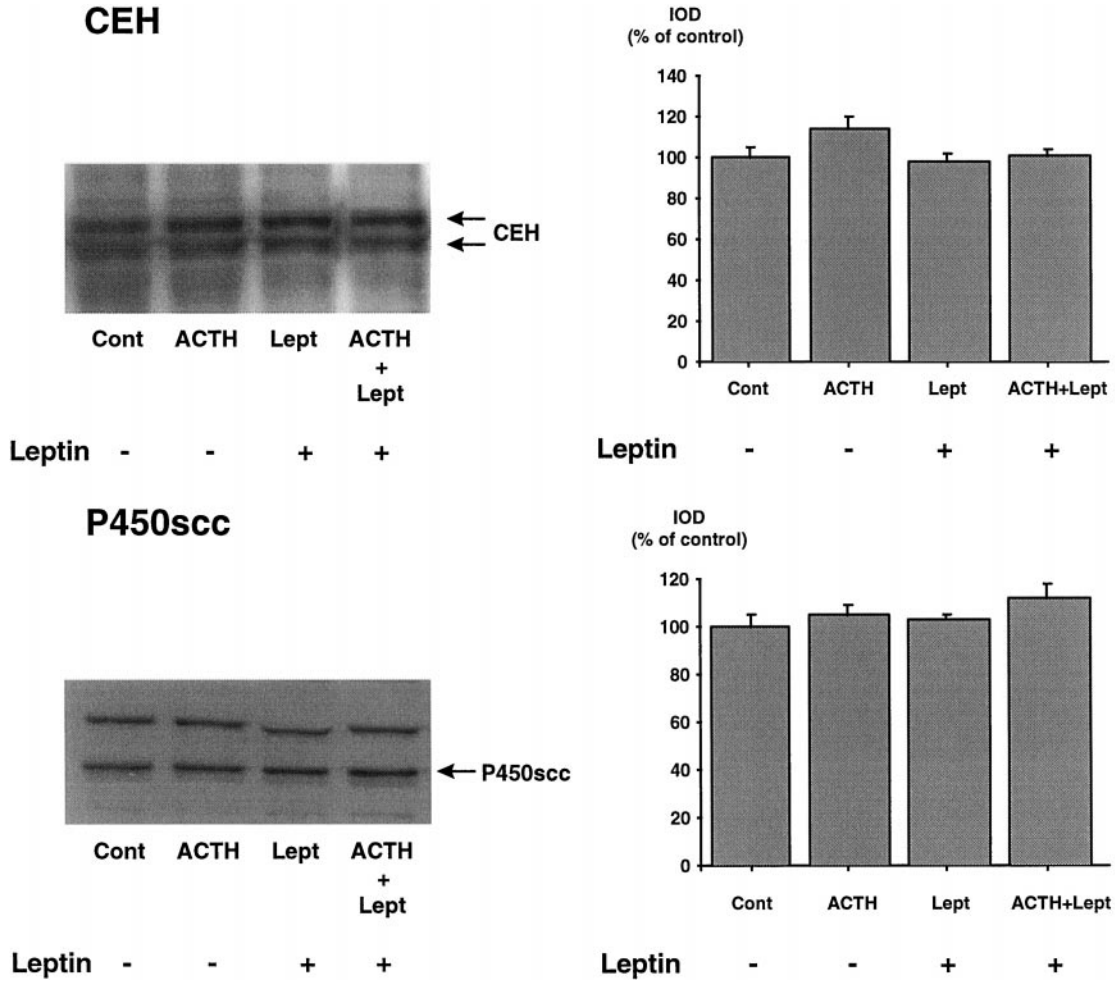

FIG. 2. Leptin does not affect CEH or $P450_{scc}$ enzyme expression. Adrenocortical cells were preincubated for 24 h in the absence (−) or presence (+) of leptin (10^{-7} M). Incubation media were changed, and cells were further stimulated with ACTH (10^{-9} M), leptin (10^{-7} M), or both for 90 min. Total cell extracts (10 mg protein) were prepared and analyzed by SDS-PAGE and immunoblotting for the CEH (protein doublet of 84 and 89 kDa; *upper panel*) and the $P450_{scc}$ (49 kDa; *lower panel*) enzymes. A nonspecific immunoreactive band with an apparent M_r of 60 kDa was revealed with the $P450_{scc}$ antiserum. The Western blots shown are representative of three independent experiments. Each value in the bar graphs is the mean ± SEM of triplicate determinations from three independent experiments. IOD, Integrated optical density.

could be involved in a down-regulation of that axis (23). Recent evidence suggests that this modulation is probably operational at the level of both the hypothalamus (5, 13) and the adrenal gland, but studies investigating the direct effects of leptin on CRH secretion have led to conflicting results (15, 16, 36). In the adrenal gland leptin has been shown to inhibit cortisol or corticosterone secretion from bovine (18), rat (17), as well as human (17, 37) adrenocortical cells *in vitro*, thus modulating net cortisol or corticosterone output. However, existing data on this direct effect of leptin are somewhat conflicting as well, as acute leptin treatment *in vitro* has also been reported to stimulate corticosterone (38) or dehydroepiandrosterone sulfate production (39).

These apparent discrepancies can probably be at least partially ascribed to differences in the time course of the applied stimulus (38), or differences between the experimental paradigms. Indeed, the stimulation of dehydroepiandrosterone sulfate secretion by leptin was reported in different tumor cell lines (39), and such models may exhibit responses that diverge notably from those observed in primary cells in culture. In the present study we were able to demonstrate for the first time that the physiological induction of StAR protein by ACTH, a rate-limiting step in adrenal glucocorticoid biosynthesis (26, 34), is significantly reduced by leptin treatment. These results confirm the predominantly inhibitory effects that leptin exerts on corticosterone production in primary rat adrenocortical cells (17, 18, 40). The data indicate that leptin can counteract ACTH-stimulated steroidogenesis by preventing the hormone-induced increase in StAR mRNA steady state levels. Thus, the present work highlights a novel mechanism of action for leptin in this peripheral modulation. The rather long time course required for leptin to exert this inhibition is compatible with an effect mediated at the level of gene and protein expression. However, further work will be necessary to elucidate the signaling pathway involved in this effect of leptin as well as the molecular mechanism(s) responsible for the late blunted response of StAR to ACTH reported here. It is noteworthy that no inhibition was seen when leptin was added together with ACTH. At present, one can only speculate that ACTH stimulation at the time of

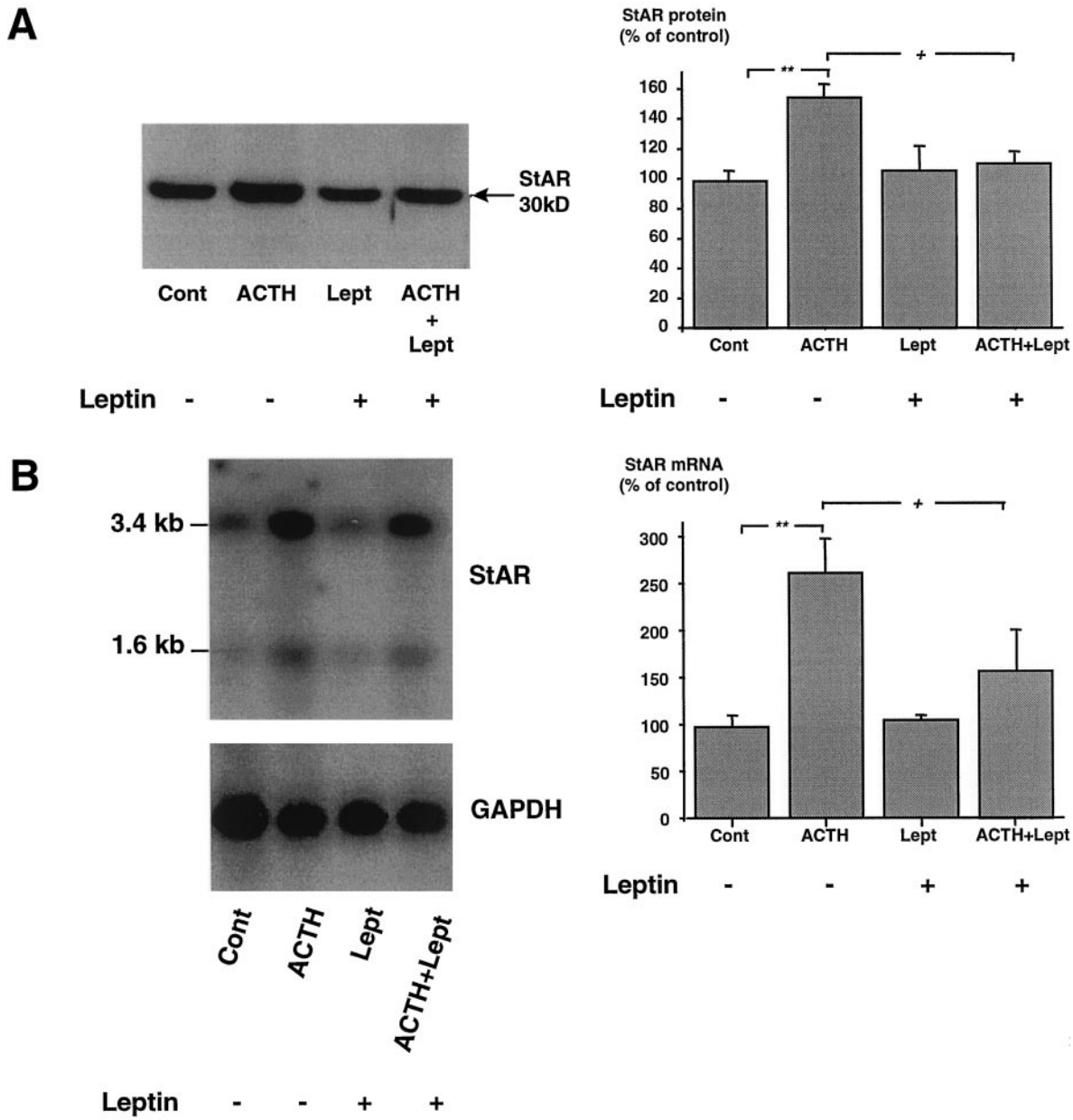

FIG. 3. Leptin (10^{-7} M) inhibits the ACTH (10^{-9} M)-induced increase in StAR protein and mRNA steady state levels. A, Adrenocortical cells were treated as reported in Fig. 2 and were analyzed by immunoblotting for their StAR protein content (*left panel*). The *right panel* illustrates densitometric analysis of three independent experiments. **, Significantly different from control, $P < 0.01$; +, significantly different from ACTH, $P < 0.05$. B, Adrenocortical cells were preincubated for 24 h in the absence (−) or presence (+) of leptin before exposure to ACTH or leptin or both for 90 min. Total RNA was isolated and analyzed by Northern blotting (*left panel*). Densitometric quantification of StAR mRNA levels in three independent experiments is shown in the *right panel*. The results were normalized to GAPDH mRNA levels, and the corrected values were expressed as a percentage of StAR mRNA levels in control cells. **, Significantly different from control, $P < 0.01$; +, significantly different from ACTH, $P < 0.05$.

exposure to leptin can override the inhibition induced by leptin pretreatment, because normal production of the StAR protein is immediately elicited, before the occurrence of any effect of leptin.

Leptin has been shown to affect various steps in adrenal glucocorticoid biosynthesis, decreasing the expression of $P450_{C21}$ hydroxylase, $P450_{scc}$, and $P450_{c17\alpha}$ hydroxylase (18, 24). More recently, a leptin-induced down-regulation of the expression of the leptin receptor in the adrenal gland was demonstrated (40). In the latter study the researchers (40) could confirm that leptin inhibits corticosterone secretion from rat adrenal slices. In addition, these researchers were able to demonstrate that leptin can desensitize the adrenal gland to its own effects by decreasing the expression of the leptin receptor, Ob-R. In parallel with these effects of leptin, ACTH itself was found to reduce the adrenal expression of all isoforms of Ob-R in that study (40), thus providing a limitation to the inhibition of its own effects induced by leptin. Therefore, it seems that leptin and ACTH probably interact closely in several different ways to achieve a fine-

tuning of the overall stress response (21–23). In addition, by providing a novel intracellular target for the effects of leptin, the present data add to our general understanding of the bidirectional relationships existing between leptin and the HPA (stress) axis (21).

Overall, this effect of leptin on the adrenal gland could be relevant to human pathophysiology. Indeed, the sometimes markedly elevated leptin levels observed in human obesity (41) could induce significant alterations in the adrenal responsiveness to ACTH stimulation. If this were true, it could lead to abnormal feedback of glucocorticoids on the hypothalamo-pituitary unit, eventually resulting in a disruption of the circadian rhythm. Such an abnormal regulation of the HPA axis can be seen in obesity. This adrenal effect of leptin could also be important in critically ill septic patients. In these patients, elevated leptin levels and, incidentally, a loss of the diurnal rhythm of cortisol have been associated with a better clinical outcome (42). It could be hypothesized that these elevated leptin levels act to decrease the stress response, thus allowing a more efficient immune response to take place. Finally, we previously suggested that an abnormal sensitivity of the adrenal glands to leptin may participate in the pathogenesis of the rare form of food-dependent adrenal Cushing's syndrome seen in macronodular adrenal hyperplasia (43). The hypothesis that the StAR protein, a rate-limiting factor in adrenal glucocorticoid biosynthesis, could be involved in this paradoxical response to leptin probably warrants further investigation.

In conclusion, we demonstrated that the StAR protein participates in the physiological down-regulation of adrenocortical function by leptin. This leptin-dependent fine-tuning of adrenal function could be of clinical relevance in obesity and related disorders as well as in the pathogenesis of rare adrenal tumors. Further work will be necessary to clarify the molecular mechanisms responsible for the effects of leptin on StAR protein expression described here.

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Address all correspondence and requests for reprints to: François P. Pralong, M.D., BH 19-707, University of Lausanne Medical School, CH-1011 Lausanne, Switzerland. E-mail: francois.pralong@chuv.hospvd.ch.

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New Formatting for *Endocrinology*

In this issue of *Endocrinology* you will notice several formatting changes that we hope will be helpful to you as readers. As directed by the Publications Committee and finalized in collaboration with the other Endocrine Society journals, these changes are intended to provide a more cohesive look for the Society's publications, as well as to make the journals easier to read and cite. While many of these changes are subtle, we thought it best to point them out to you.

The list of Standard Abbreviations (July 2001) has been updated and standardized for all four journals. Standard Abbreviations will now be used in the titles of papers, while no nonstandard abbreviations will be allowed in the text of the Abstract. Nonstandard abbreviations will be defined in the text and in a definition footnote that will appear on the first page of every article. All page headers will now contain full citation information, as well as the name of the first author and the abbreviated title. Acknowledgments, including reprint and correspondence contacts as well as dates of receipt and acceptance, will be published at the end of each article. Finally, references may now list up to six authors; for references with seven or more authors we will list the first three and add "*et al*".

We hope you find these formatting changes make *Endocrinology* a better resource for you.

Kenneth S. Korach
Editor-in-Chief