ACTH Decreases the Expression and Secretion of Apolipoprotein B in HepG2 Cell Cultures*

Ning Xu, Ulf Ekstrom, and Peter Nilsson-Ehle‡

From the Department of Clinical Chemistry, Institute of Laboratory Medicine, University Hospital of Lund, S-221 85 Lund, Sweden

Administration of adrenocorticotropic hormone (ACTH) has been shown to decrease plasma concentrations of apolipoprotein B (apoB) containing lipoproteins, including lipoprotein(a), in man. However, the mechanism behind this hypolipidemic effect is unknown. This study aimed at distinguishing between the main possibilities (increased elimination or decreased production of lipoproteins) using HepG2 cell cultures. Addition of ACTH to the cell culture medium selectively down-regulated apoB mRNA expression and apoB secretion in a dose-dependent manner. At 100 pmol/liter ACTH, the apoB mRNA level was about 40% lower than in the untreated cells, and the secretion of apoB into the medium was decreased to a similar extent. The expression and secretion of other apolipoproteins (apoA-I, apoE, and apoM), however, were not affected by ACTH. Under normal culture conditions the level of secretion of apoB from HepG2 cells is quite low. In the presence of 0.4 mmol/liter oleic acid secretion of apoB increased 3-fold, but this phenomenon was not seen in ACTH-treated cells. Binding and internalization of radiolabeled low density lipoprotein (LDL) by HepG2 cell, as well as LDL-receptor mRNA and scavenger receptor B-I mRNA levels, were not influenced by ACTH. In conclusion, ACTH directly and selectively down-regulated the production and secretion of apoB in HepG2 cell cultures, suggesting that a principal mechanism behind the cholesterol-lowering effect of ACTH in vivo may be a decreased production rate of apoB-containing lipoproteins from the liver.

It is well documented that adrenocorticotropic hormone (ACTH)³ regulates important aspects of lipid metabolism in the adrenal gland, especially those related to steroid hormone homeostasis (1). ACTH has also been shown to influence plasma lipid and lipoprotein metabolism (2–7). Thus, we have demonstrated previously that ACTH treatment markedly and consistently lowers plasma concentrations of low density lipoproteins (LDL), very low density lipoprotein (VLDL), and lipoprotein(a) (Lp(a)) in healthy individuals (2, 7), in steroid-treated patients with renal disease, and in uremic patients treated by hemodialysis (3, 4). In some of the studies, an increased high density lipoprotein (HDL) cholesterol concentration was also observed (4, 6). The effects of ACTH on LDL and Lp(a) levels seem attributable to ACTH as such, because they are clearly distinct from the effects of equipotent doses of corticosteroids (6).

The mechanisms behind the lipid-lowering effects of ACTH are not known. Previous data indicated that ACTH could increase LDL receptor (LDL-R) activity in HepG2 cells (6), suggesting that an increased elimination rate of LDL might be involved. It has also been reported that ACTH increased the receptor-mediated uptake of native LDL but not of oxidized LDL or of Lp(a) in HepG2 cells. However, because the elimination of LDL and Lp(a) from the circulation occurs via distinct different routes (i.e. Lp(a) does not utilize the LDL-R pathway), the parallel decrease in plasma LDL and Lp(a) concentrations after administration of ACTH in vivo indicates that other mechanisms must also be involved. Because apolipoprotein B (apoB) is the main protein component of both LDL and Lp(a), an obvious possibility is that ACTH would affect the production of lipoproteins in the liver rather than their removal from plasma.

In the present study, therefore, we investigated the effects of ACTH on the expression and secretion of apoB and other apolipoproteins and re-examined the influence of ACTH on the receptor-mediated uptake of LDL in HepG2 cell cultures. Because LDL removal may occur not only by the LDL-R but also by the scavenger receptor BI (SR-BI) (9, 10), we monitored the effects of ACTH on the expression of these two receptors.

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‡ To whom correspondence should be addressed. Tel.: 46-46-173452; Fax: 46-46-130064; E-mail: peter.nilsson-ehle@klinikum.lu.se.
³ The abbreviations used are: ACTH, adrenocorticotropic hormone; LDL, low density lipoprotein; VLDL, very low density lipoprotein; Lp, lipoprotein; HDL, high density lipoprotein; LDL-R, LDL-receptor; apo, apolipoprotein; SR-BI, scavenger receptor BI; FCS, fetal calf serum; PBS, phosphate-buffered saline; HSA, human serum albumin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair.

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**ACTH Down-regulates apoB Production**

**Preparation of Northern Blotting Probes**—A commercial G3PDH cDNA fragment or a β-actin cDNA fragment (CLONTECH) was used as a control probe. A full-length human apoM cDNA was used as probe for apoM mRNA hybridization (12). A 325-bp DNA fragment corresponding to apoB LDL-R binding domain was amplified by polymerase chain reaction (primers, 5'-gtgctactatcaagaag and 5'-gcattgtcaggtgagag) from human genomic DNA and used as human apoB Northern blotting probe. A 651-bp apoA-1 exon 3 DNA fragment was amplified from human genomic DNA (primers, 5'-ctggagagggtgcctg and 5'-tctgggacagcgtacctc) as a probe for apoA-1 hybridization (11). To be used as a probe for apoE hybridization, a 317-bp DNA fragment of the apoE gene was amplified from human genomic DNA (primers, 5'-ctgttcagccagcatgaa and 5'-tccgggggccccggcctggta). A 932-bp DNA fragment of the LDL-R gene (exons 12–18) was amplified from human genomic DNA (primers, 5'-tatcagctgtactctaccgctaaagga and 5'-gagagagtt) from human genomic DNA and used as human apoB Northern blotting probe (13). To be used as an LDL-R probe. A 195-bp fragment was amplified from exon 1 of the gene coding for SR-B1 (primers, 5'-cgctgcttgcctctcgagc and 5'-cttgaagcgtctgtagagc) as a probe for SR-B1 (14). All polymerase chain reactions were performed using AmpliTaq DNA polymerase with buffers and dNTPs supplied by manufacturer (PerkinElmer Life Sciences) according to the manufacturer's directions, on a GeneAmp polymerase chain reaction system 2400 (PerkinElmer Life Sciences). All probes were radiolabeled with 32P[α-32P]ATP using the random primer method (RediPrime; Amersham Pharmacia Biotech).

**Isolation of Total RNA from HepG2 Cells and Northern Blotting Analysis**—Total RNA of HepG2 cells was isolated by the guanidinium thiocyanate method, and Northern blots were hybridized with the probes described above and eventually exposed to x-ray film (15). G3PDH or β-actin probes were used as controls. Hybridizations were carried out at 65 °C in a hybridization solution (CLONTECH) with 35S-labeled DNA probes. The blots were washed several times in 2× SSC/0.1% SDS solution at room temperature for 2 h and twice in 0.1× SSC/0.5% SDS at 50 °C for 40 min. The washed blots were exposed to x-ray film at ~70 °C from 1 day to 1 week. The autoradiographs were analyzed with a scanner (UMAX Astra 1220U; UMAX Data Systems Inc.). The membrane was stripped with boiled water in the presence of 0.5% SDS for 10 min and then rehybridized with the next probe. To compare the effects of ACTH on different apolipoprotein and lipoprotein receptor mRNA levels, all Northern blotting analyses, except for LDL-R, were performed using the same membrane. LDL-R mRNA levels were determined in a separate series of experiments, using many different membranes. The relative mRNA levels were calculated with a Macintosh computer using Quantity One software (version 4.2.1; Bio-Rad).

**Apolipoproteins, B, A-1, E, and M Protein Mass Determinations**—apoB, apoA-1, apoE, and apoM masses were determined by Western blotting analysis. Cell culture medium without FCS was fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated with rabbit polyclonal antibodies against human apoA-1, apoE, or apoM. Horseradish peroxidase-conjugated IgG was used as the secondary antibody. Bands corresponding to the different apolipoproteins were visualized by an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech) or developed by the peroxidase staining method and quantified by a scanner using Quantity One software.

**Preparation of Radiolabeled LDL and Determination of LDL-R Activity**—Human plasma LDL was separated and labeled with [125I] by the monochloride method according to Raynes et al. (16). The LDL-R activity was measured by determination of the binding and internalization of radioactive labeled LDL by HepG2 cells. In brief, the subconfluent HepG2 cells were incubated with the experimental medium containing 0, 10, or 100 pmol/liter ACTH. The binding and uptake of LDL by HepG2 cells were studied at 4 and 37 °C, respectively. To estimate binding, the cells were washed three times with 1 ml of ice-cold PBS and preincubated for 20 min on ice with 2 ml of ice-cold medium without HSA. The cells were then incubated at 4 °C for 2 h with [125I]LDL (9.86 µg/ml) and subsequently washed three times with ice-cold PBS. 2 ml of dextran sulfate (10 mg/ml) were added, followed by a 1-h incubation on a shaker at 4 °C. The supernatant was separated, and radioactivity was counted using a gamma counter (LKB 1280, multiwindow gamma counter; Wallac Service). Finally the wells were scraped after addition of 2 ml of 0.5 M NaOH, and the protein content of the cells were determined according to the method described by Lowry et al. (17). The LDL uptake (binding plus internalization) by HepG2 cells was examined at 37 °C. The cells were washed three times with 1 ml of PBS, incubated for 2 h with [125I]LDL (9.86 µg/ml), and washed three times with PBS. After addition of 2 ml of 0.5 M NaOH, the cells were scraped off the wells, and the radioactivity was measured in a γ counter. Protein content was measured by the method described by Lowry et al. (17).

**Statistics**—Results are expressed as mean ± S.D. Comparisons among groups were statistically evaluated by the Mann-Whitney U test. Significance was established at a p value less than 0.05.

**RESULTS**

**Effect of ACTH on the Secretion of Apolipoproteins from HepG2 Cells**—Fig. 1 demonstrates the contents of apoB in the medium from cell cultures exposed to ACTH for 6, 12, and 24 h, respectively. ACTH significantly lowered apoB concentrations at all three time intervals. The effect was most pronounced at higher concentrations of ACTH (50 and 100 pmol/liter), indicating that ACTH influences apoB production in a dose-dependent manner. In another series of experiments, we treated cells once with ACTH (100 pmol/liter) and followed the concentrations of apoB in the medium by repeated sampling over time. A decrease of apoB levels occurred after 3 h and reached maximum after 24 h (Fig. 2). In the presence of 0.4 mmol/liter oleic acid the level of secretion of apoB in control cell cultures increased about 3-fold, but this phenomenon was not seen in ACTH-treated cells (Fig. 3). Thus, oleic acid could not reverse the inhibitory effect of ACTH on apoB secretion. There were no significant changes in the production of apoA-1, apoE, and apoM in the medium after ACTH administration (data not shown). There were no morphological changes in cells after ACTH treatment (data not shown).

**Effect of ACTH on Apolipoprotein mRNA Levels in HepG2 Cells**—Fig. 4 demonstrates that ACTH significantly suppressed apoB mRNA expression, as determined by Northern blotting analysis, in a dose-dependent manner. At 100 pmol/liter, the reduction in apoB mRNA was about 45% compared with control mRNA. This effect of ACTH was selective for apoB mRNA, because there was no corresponding decrease in the mRNA of other apolipoproteins or in the control mRNA, i.e. G3PDH mRNA (Fig. 4).

**Effects of ACTH on Receptor-mediated Uptake of LDL in HepG2 Cells**—Stimulation of HepG2 cells by ACTH did not influence the receptor mediated uptake of LDL, as determined
by binding or uptake of radioactively labeled LDL. Numerous experiments were performed, but no consistent or significant effects on LDL binding or uptake could be documented at various time points, concentrations, or other relevant manipulations of the experimental conditions. Data from representative experiments are given in Table I.

Effects of LDL-R and SR-BI mRNA Levels in HepG2 Cells—Because the uptake of the radiolabeled LDL in cell cultures might be influenced by alterations in the medium occurring during the incubations (e.g. secretion of apoB or other apolipoproteins into the medium), we also investigated the possible effects of ACTH on the levels of LDL-R and SR-BI mRNA. As demonstrated in Fig. 4 and Fig. 5, ACTH did not influence the expression of the LDL-R or SR-BI in HepG2 cells.

**DISCUSSION**

In a series of investigations in healthy volunteers and in patients with moderate secondary hyperlipoproteinemia due to renal diseases, we have demonstrated that administration of ACTH strongly and consistently lowers plasma cholesterol and triglyceride levels in humans (2–7). The concentrations of apoB-containing lipoproteins VLDL, LDL, and LP(a) are markedly reduced, whereas plasma HDL levels are unaffected or increase moderately (2–7). Largely, these effects, notably those on apoB-containing lipoproteins, seem to be mediated via ACTH per se, because administration of corticosteroids under similar conditions tends to increase plasma lipid concentrations (6). The present study supports the view that ACTH exerts direct effects on lipid metabolism, demonstrating that addition of ACTH to HepG2 cell cultures affects key events in lipoprotein metabolism in liver cells. Although it is generally considered that ACTH largely acts indirectly, via cortisoloids, direct metabolic effects of ACTH have been suggested previously, because ACTH promotes the mobilization of fatty acids from adipose tissue in the rat (18).

The mechanisms behind the effects of ACTH on lipoprotein metabolism in vivo are still obscure. Theoretically, the hypolipidemic effects could be the result of an inhibition of the production of lipoproteins in the liver or of enhanced catabolism of LDL particles via lipoprotein receptor-mediated pathways. The present study, using HepG2 cell cultures, supports the view that ACTH primarily affects lipoprotein production, demonstrating that ACTH decreased significantly apoB mRNA levels in hepatic cells and apoB secretion into the medium, whereas there was no effect of ACTH on receptor-mediated LDL uptake or on LDL-R mRNA and SR-BI mRNA concentrations. ApoB is the structural component of VLDL and LDL. Expression of apoB determines VLDL production and LDL-LDL cholesterol during administration of ACTH in vivo.

We have reported earlier that ACTH could up-regulate LDL-R activity in HepG2 cells (6). Similarly, Bartens et al. (8) reported that ACTH could stimulate LDL-R activity in HepG2 cells, promoting the uptake of labeled native LDL but not that of oxidized LDL or Lp(a). In the present study, we were not able to reproduce these data. As shown in Table I, numerous exper-
we found that ACTH had no effect on SR-BI mRNA in liver cells.

Table I

| ACTH (pmol/liter) | LDL-R binding | LDL-R binding + internalization |
|-------------------|---------------|---------------------------------|
|                   | 0  | 10 | 100 | 0  | 10 | 100 |
| **Experiment 1**  |    |    |     |    |    |     |
| 26 ± 1            | 23 ± 0 | 24 ± 5 | 153 ± 5 | 172 ± 16 | 148 ± 17 |
| 39 ± 6            | 32 ± 2 | 34 ± 3 | 90 ± 7 | 87 ± 9 | 93 ± 5 |
| **Experiment 3**  |    |    |     |    |    |     |
| 23 ± 3            | 25 ± 1 | 22 ± 2 | 85 ± 6 | 81 ± 1 | 84 ± 4 |
| **Experiment 4**  |    |    |     |    |    |     |
| 24 ± 1            | 35 ± 3 | 27 ± 1 | 100 ± 9 | 111 ± 4 | 111 ± 2 |

**Fig. 5. Effect of ACTH on LDL-R mRNA expression.** Extraction of total RNA and Northern blotting analysis were as described in the legend for Fig. 4. β-Actin was used as a control probe. Aliquots of 10 μg of total RNA isolated from control cells without ACTH (lane 1), cells treated with 50 pmol/liter ACTH (lane 2), and cells treated with 100 pmol/liter ACTH (lane 3) were used for Northern blot analysis. Data are mean ± S.D. (n = 6 for each sample group). Results are representative of six similar experiments.

SR-BI in hepatic cells or in adrenals are not involved in the ACTH-lowering cholesterol effect observed in vivo.

The availability of lipids is critical in determining apoB particle assembly and secretion. Dixon et al. (22) demonstrated that oleic acid could stimulate secretion of apoB-containing lipoprotein from HepG2 cells by inhibiting early intracellular degradation of apoB (22). In the present study we also cultured HepG2 cells in the presence of 0.4 mmol/liter oleic acid with (100 pmol/liter) or without ACTH. Supplementation with oleic acid strongly stimulated apoB secretion from HepG2 cells in the absence of ACTH but not in ACTH-treated cells (Fig. 3).

Thus, the effect of ACTH could not be blocked by addition of oleic acid, which is consistent with a direct and unique inhibitory effect on apoB synthesis. However, the fact that ACTH did indeed abolish the increase in apoB secretion induced by oleic acid might indicate that ACTH could also affect lipoprotein secretion at a later stage in the secretory process, i.e. by modulating intracellular degradation of apoB.

The apoB-containing pre-VLDL particle is formed during the translation and translocation of apoB to the lumen of the rough endoplasmic reticulum (23–25) and further modified during a short period post-translationally (26) to form a mature VLDL particle that is secreted into plasma. Synthesis of apoB is the rate-limiting step in this process (18). VLDL is then transformed into LDL particles by delipidation of its lipid core by lipoprotein lipase (27–30). Thus, down-regulation of apoB synthesis may explain the reduction of VLDL, as well as of LDL, in response to ACTH.

Alterations in apoB production may be the result of reduced apoB gene transcription or enhanced apoB mRNA degradation (31, 32). The present experiments do not distinguish between these possibilities. Pulse-chase experiments using apoB gene transfected cells may help to further elucidate the effects of ACTH on VLDL production and assembly.

Our previous studies have shown that ACTH also decreases plasma Lp(a) concentration (2, 3). Lp(a) is composed of an LDL particle, which also contains apo(a). The latter seems to bind to the apoB moiety of VLDL/LDL in the circulation (33, 34). Thus, the reduction of plasma Lp(a) may be secondary to the decrease of VLDL and LDL concentrations after ACTH administration. However, it cannot be ruled out that ACTH may also affect the production of apo(a) or the elimination of Lp(a) from plasma.

In a parallel study of healthy individuals and hemodialysis patients (35), administration of ACTH also induced an increase in the serum concentrations of apoE, an observation that may hypothetically offer an alternative mechanism for the effect of ACTH on lipoprotein metabolism. ApoE is present in all lipoprotein classes and plays a central role in lipoprotein catabolism because of its high affinity to lipoprotein receptors (36). In the rabbit, intravenous injection of apoE was associated with

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a transient lowering of plasma cholesterol concentrations (37), an effect that did not involve an up-regulation of LDL-R activity (38). However, in HepG2 cells we did not observe an enhanced apoE mRNA expression or apoE secretion into the medium in response to ACTH. Nevertheless, these results do not rule out the possibility that apoE is involved in the lipid-lowering effect of ACTH in vivo, because apoE is expressed in several organs besides the liver (39).

In conclusion, the present study shows that ACTH down-regulates hepatic apoB mRNA expression and apoB secretion, which may constitute a major mechanism behind its strong and consistent lipid-lowering effect in vivo. A reduced synthesis of apo(a) containing lipoproteins may also explain the reduction of Lp(a) concentration, but further studies are needed to investigate possible effects of ACTH on the metabolism of apo(a).

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