Proteolysis and Lipid-facilitated Translocation Are Distinct but Competitive Processes That Regulate Secretion of Apolipoprotein B in Hep G2 Cells*

(Received for publication, July 16, 1993)

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Under lipid-poor conditions, most newly synthesized apolipoprotein B100 (apoB) undergoes rapid degradation in Hep G2 cells such that only a small fraction of newly synthesized apoB is actually secreted. Addition of oleate to Hep G2 culture medium stimulates apoB secretion by a post-translational mechanism. In the current studies we have explored oleate-simulation of apoB secretion by using calpain inhibitor I, N-acetyl-leucyl-leucyl-norleucinal (ALLN), a compound that inhibits the intracellular degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the T cell receptor subunit. Preincubation of Hep G2 cells with ALLN (40 μg/ml) for 1 h markedly inhibited degradation of newly synthesized apoB. Whereas only 32% of newly labeled apoB remained intact (cells + medium) in control cells after a 10-min pulse with [3H]leucine followed by a 60-min chase, 84% of labeled apoB was intact in ALLN-treated cells. However, most of the ALLN-protected apoB remained intracellular, as ALLN did not stimulate the rate of apoB secretion over the control rate (12 versus 9.2%). Although secretion of apoB was not accelerated, the protection afforded by ALLN continued for several hours, and labeled apoB continued to be secreted over 3 h of chase after which secretion ceased. The protection afforded by ALLN resulted in 37% of labeled apoB secreted by 3 h compared to 15% in control cells. In contrast, simultaneous treatment of cells with ALLN and oleate both accelerated and increased total apoB secretion, such that 36% of initially labeled apoB was recovered in the medium by 60 min and 71% of labeled apoB was secreted by 180 min of chase. These data show that ALLN and oleate affect apoB metabolism by different mechanisms. Although ALLN can protect nascent apoB from rapid early intracellular degradation, it does not accelerate apoB secretion. In contrast, although our results cannot rule out the possibility that oleate may directly inhibit proteolysis of apoB, oleate appears to prevent apoB mainly by facilitating transport of apoB out of a protease-containing compartment associated with the endoplasmic reticulum.

Apolipoprotein B (apoB), a 514-kDa glycoprotein, is required for hepatic assembly and secretion of very low density lipoprotein (1–3). Increased rates of secretion of apoB-containing lipoproteins from liver appear to be a common abnormality in individuals with hyperlipidemia (4–7). It is therefore of interest to define the regulatory mechanisms involved in the hepatic secretion of apoB-containing lipoproteins. Although changes in the level of apoB mRNA have been observed in cultured hepatocytes and in animal studies under some conditions (8–12), most previous studies have suggested that rapid changes in apoB secretion are not associated with significant changes in the level of apoB mRNA (13–20). Thus, it is believed that regulation of apoB secretion is primarily post-translational.

Despite the fact that apoB contains no classical transmembrane regions, kinetic studies indicate that after synthesis, apoB is first associated with the ER membranes (21–25), and that segments of apoB are exposed on the cytosolic surface of the ER (26–29). Once apoB is released from the ER membrane, it appears to be rapidly transported from the ER lumen to the outside of the cell via the Golgi lumen (24). Not all apoB is secreted by hepatocytes, however (30). We (28, 31) and others (32) have shown in Hep G2 cells that a significant proportion of newly synthesized apoB is rapidly degraded intracellularly. Recently, we confirmed that the ER is the site of early apoB degradation in Hep G2 cells (28).

Börn et al. (33) suggested that apoB that is targeted for secretion is cotranslationally associated with core lipids to form a nascent lipoprotein. Their hypothesis is compatible with the marked reduction in apoB degradation that is observed when oleate is added to the culture medium (31). Thus, translocation of apoB into the ER lumen might be facilitated by newly synthesized lipids, reducing availability of the protein for degradation by a protease (or proteases) located either in the cytosol or on the cytosolic side of the ER membrane (26, 28). Indeed, we showed that addition of oleate to the culture medium appears to reduce the availability of newly synthesized apoB for degradation by proteinase K in an in vitro assay using isolated microsomes (28).

The calpain inhibitor I, N-acetyl-leucyl-leucyl-norleucinal (ALLN), is a potent inhibitor of the degradation of both 3-hydroxysterol-methylglutaryl-coenzyme A and the T cell receptor subunit in the ER (34). Thrift et al. (35) recently demonstrated that ALLN inhibits the degradation of a truncated form of apoB (apoB53) in Chinese hamster ovary fibroblasts. These cells do not, however, have the capacity to secrete apoB (35), so these investigators could not determine the effects of ALLN on the assembly and secretion of apoB-containing lipoproteins. In the present study we have compared the effects of ALLN and oleate on apoB degradation and secretion in Hep G2 cells. Our goal

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*This work was supported by Grants HL-38600, HL-21006, and HL-47586 from the National Heart, Lung, and Blood Institute, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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was to differentiate the effects of direct inhibition of protease-associated degradation of apoB from the effects of lipid-stimulated translocation of the protein. We found that direct inhibition of proteolysis by ALLN treatment alone is associated with a marked increase in an intracellular pool of nascent apoB that is very slowly secreted from the cell. Addition of oleate to ALLN-treated Hep G2 cells results in both markedly increased rates of and greater total apoB secretion. These studies provide evidence that in native Hep G2 cells (in the absence of ALLN), proteolysis and lipid-facilitated translocation are distinct, competitive processes that regulate apoB secretion. Furthermore, our results indicate that translocation of nascent apoB away from a proteolytic enzyme is the critical step in the post-translational stimulation of apoB secretion by oleate.

**EXPERIMENTAL PROCEDURES**

**Materials—**I-leucine was purchased from Amersham Corp. Monospecific anti-human apoB antibody was raised in a rabbit. Protein A-Sepharose CL-4B was obtained from Pharmacia LKB Biotechnology Inc. Minimum essential medium, nonessential amino acid, sodium pyruvate, and penicillin/streptomycin were from Life Technologies, Inc. Labeling of cells was performed with leucine-free medium generated from a minimum essential medium Select-amine kit, which was purchased from Life Technologies, Inc. Bovine serum albumin (BSA) (essentially fatty acid-free) and oleic acid (sodium salt) were purchased from Sigma. 

**Immunoprecipitation**—Immunoprecipitation of apoB in medium and cells was performed as previously described (28, 31). Briefly, the cells (1 x 10⁶ cells) were seeded into each well of the six-well tissue culture plate (31). After incubation, the tissue culture plates were covered with a thin layer of medium containing PHIleucine (3H) and chased with serum-free medium for 60 min. After the preincubation, the cells were washed twice with PBS and preincubated with serum-free medium for 60 min. After preincubation, the cells were washed twice and harvested in lysis buffer by scraping. 

**RESULTS AND DISCUSSION**

**ALLN Inhibits ApoB Degradation but Does Not Stimulate Short Term Secretion**—In order to define the effects of ALLN on newly synthesized apoB, Hep G2 cells were labeled with 3Hleucine for 10 min and chased for 60 min in the absence or presence of 40 μg/ml ALLN. Preliminary studies had demonstrated that 40 μg/ml ALLN did not affect total protein synthesis or the secretion of either albumin or apolipoprotein A-I in Hep G2 cells (data not shown). However, concentrations greater than 50 μg of drug/ml significantly decreased protein synthesis.

In the absence of ALLN (control cells) after 60 min of chase (Fig. 1), the cellular level of apoB had dropped to 23.0% of initially labeled apoB while only 9.2% of labeled apoB was secreted into the medium. These data indicated that 68% of newly synthesized apoB was rapidly degraded during the 60 min of chase. In the presence of ALLN, degradation of apoB was markedly reduced as 84% of newly labeled apoB remained intact (cellular plus medium) after 60 min (Fig. 1). The secretion of apoB, however, was not increased significantly by ALLN (12% of labeled apoB was secreted by 60 min of chase). These results support the recent finding that the degradation of truncated apoB (apoB53) was sensitive to ALLN in CHO cells (35). CHO cells, however, cannot secrete intact apoB53, and our results, therefore, are the first demonstration of an effect of ALLN in hepatic cells.

In contrast to the effects of ALLN, secretion of apoB from Hep G2 cells was increased significantly during the 60-min chase period in the presence of 0.4 μg/ml oleate (21% of initially labeled apoB was secreted), even though apoB degradation was not reduced as much as observed with ALLN (only 55% of total labeled apoB remained intact after 60 min of chase with oleate) (Fig. 1). These effects of oleate on apoB degradation and secretion were similar to our previous findings (28, 31). The data suggested that the stimulatory effect of oleate on apoB secretion could not be explained fully by a mechanism involving direct inhibition of apoB degradation. The results indicated that the effects of ALLN and oleate might involve different regulatory processes.

**ALLN Increases the Pool of ApoB with Potential for Secretion, but Core Lipids Are Needed for Efficient Secretion**—The next experiment was designed to determine if ALLN-protected apoB retained the capacity to be secreted over longer chase periods. Therefore, we measured the accumulation of labeled apoB in medium of control and ALLN-treated cells at varying time points during a 3-h chase period. In control cells, only 15% of newly synthesized apoB was secreted by 180 min (Fig. 2A). Examination of the time course of accumulation of apoB in the medium indicated that, in the absence of ALLN, the major portion of apoB destined for secretion exited the cell within the first 60 min of chase. By 120 min, secretion of labeled apoB from control cells was complete. These data are depicted as secretion rates in Fig. 2B. The percentage of initially labeled apoB that was secreted per minute in control cells was 0.16 ± 0.04% (mean ± S.D.) during the first hour. In the second hour, this rate decreased to 0.03%/min and was unchanged thereafter. In contrast, while only 12% of newly synthesized apoB was secreted by 60 min in the presence of ALLN, this
ALLN treatment did not stimulate the rate of labeled apoB secretion and the rate of labeled apoB from treated and labeled cells as described in Fig. 1. B, data are presented as secretion rates of apoB and calculated as the percentage of initially synthesized apoB secreted per minute. 0–60, 60–120, and 120–180 min denote secretion rate of apoB during first, second, and third hours of the chase period, respectively. Open columns, BSA alone; diagonally hatched column, ALLN; cross-hatched column, ALLN plus oleate.

The results with ALLN alone suggested that, although direct inhibition of proteolysis increased the total pool of nascent apoB in the cells and although this protected pool of apoB retained the potential for secretion, the rate of secretion was determined independently, probably by the availability of core lipids (28, 31). This hypothesis was supported by the results obtained when Hep G2 cells were treated simultaneously with ALLN and oleate. The secretion of initially labeled apoB from cells treated with both ALLN and oleate was markedly increased to 36% by 60 min and 68% by 120 min of chase. Only slightly greater total secretion (71% of initially labeled apoB) was achieved by 180 min of chase (Fig. 2A). The secretion rates were 0.21 ± 0.07, 0.27 ± 0.1, and 0.15 ± 0.01/min in the first, second, and third hours, respectively (Fig. 2B). These data indicated that although ALLN treatment did not stimulate the rate of labeled apoB secretion significantly compared to the rate observed during the first hour of chase in control cells, it was associated with persistence of this rate and, therefore, much greater total secretion of apoB during the entire 3-h chase period.

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The addition of oleate to ALLN-treated cells clearly resulted in increased secretion of apoB from the intracellular pool appeared to be more rapid in cells treated with ALLN plus oleate, consistent with the accelerated, early secretion that was observed.

The data in Figs. 4 (A and B) were used to calculate the percent of initially labeled apoB that remained intact, either in cells or in medium, over the course of the 7-h chase (Fig. 4C). The addition of oleate to ALLN-treated cells clearly resulted in greater total recovery of apoB than was observed with ALLN alone. During the first 2 h of chase, practically no apoB was degraded in cells treated with ALLN plus oleate, whereas the majority of initially synthesized apoB was degraded in cells treated with ALLN alone. These findings can be interpreted in two ways. First, they suggest that under the current conditions ALLN treatment may not inhibit apoB degradation completely, and that oleate treatment may directly supplement the protection afforded by ALLN. Second, oleate may facilitate the transport of apoB away from a site where the protein is vulnerable to degradation (even in the presence of ALLN). In either case, it seems that the protective effect of oleate results from its ability to facilitate, or accelerate, the formation and secretion of apoB-containing lipoproteins.

In summary, our studies indicate that ALLN and oleate treatment of Hep G2 cells affect apoB secretion by different mechanisms. ALLN directly inhibits a protease that normally targets newly synthesized apoB for rapid ER degradation. In contrast, while our studies can not entirely rule out the possibility that oleate directly inhibits apoB degradation, its most
significant effect appears to be to facilitate the transport of apoB out of the ER and the cell.

What can these results tell us about the partitioning of nascent apoB between pathways of degradation and secretion in native Hep G2 cells? Our observation that ALLN treatment, which almost completely prevented the intracellular degradation of apoB, was not associated with accelerated secretion of apoB, is compatible with the proposal that newly synthesized apoB is localized in a restrictive compartment and that lipid is required to allow the advancement of apoB beyond this compartment. If nascent apoB was rapidly and completely translocated into the ER lumen under control lipid conditions, ALLN treatment should have been associated with rapid secretion of the protected pool of apoB. Although Chuck et al. (36, 37) and Pease et al. (38) have presented contrasting data concerning the mechanism regulating the initial insertion of nascent apoB into the ER membrane, Davis et al. (26), Dixon et al. (27), and Furukawa et al. (28) have demonstrated that a large proportion of newly synthesized apoB is located on the cytosolic side of the ER membrane. A model that would explain the differential effects of ALLN and oleate on intracellular apoB metabolism is one in which total translocation of nascent apoB beyond a compartment vulnerable to an ALLN-sensitive protease is not accomplished until adequate amounts of lipid become associated with apoB.

Can these studies also shed light on the role of lipid in the regulation of apoB secretion? Borén et al. (33) suggested that cotranslational addition of lipid was critical for the rapid translocation of apoB to the ER lumen and secretion. They (33) proposed that when apoB is not cotranslationally associated with core lipids, it is irreversibly targeted for degradation. Our finding that, in the presence of ALLN, oleate could stimulate apoB secretion even if added well after translation of apoB had been completed, indicates that cotranslational addition of lipid is not an absolute requirement for subsequent secretion. We believe, instead, that each newly synthesized apoB has, after partial cotranslational translocation through the ER membrane, the potential either for complete translocation and secretion or for degradation. The greater the time spent in this partially translocated state, the greater the probability that nascent apoB will be degraded by an ALLN-sensitive protease. In native Hep G2 cells, where the rate of triglyceride synthesis is low (39), the large majority of newly synthesized apoB remains partially translocated long enough for efficient degradation to occur. We believe that oleate treatment, by increasing triglyceride synthesis, reduces degradation of apoB by accelerating its translocation from the protease-containing compartment.

Acknowledgment—We thank Ludmila Karamerov for assisting with the preparation of the manuscript.

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