Fasting decreases apolipoprotein B mRNA editing and the secretion of small molecular weight apoB by rat hepatocytes: evidence that the total amount of apoB secreted is regulated post-transcriptionally

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Abstract Two different molecular weight forms of apoB are produced from a common initial transcript via editing of a Gln codon (CAA) to a stop codon (UAA), leading to a truncated translation product (apo B_S) that consists of the amino terminal half of the larger form (apoB_L). Previous studies have shown that fasting coordinately decreases lipogenesis and the secretion of very low density lipoprotein (VLDL) lipids and apoB_S. Secretion of the apoB_L is unaffected by fasting. We studied whether editing of apoB RNA is repressed by fasting, thus accounting for the selective decreased secretion of apoB_S. Column chromatography of [^{35}S]methionine-labeled lipoproteins secreted by hepatocytes from fed rats showed that essentially all of apoB_L is secreted in the VLDL fraction, whereas a significant amount (15%) of apoB_S is secreted associated as lipoproteins eluting in the HDL fractions. Fasting decreased the relative amount of apoB_S that eluted in the VLDL fractions and increased the amount secreted in the HDL fractions. Consistent with previous results, hepatocytes from fasted rats show a selective twofold decrease in apoB_S secretion. Fasting did not affect the relative abundance of apoB RNA, determined by slot blot hybridization assays using two different ^{32}P-labeled cDNA probes coding either for both molecular weight forms or for only the large molecular weight form. However, quantitation of the editing of apoB RNA showed that fasting caused a 60% decrease in the amount of apoB RNA possessing the stop codon. These data show that the editing of apoB RNA is sensitive to metabolic state (i.e., fasting) resulting in a selective decrease in the secretion of apoB_S. However, since the total secretion of apoB was decreased by fasting, while apoB mRNA levels remained constant, additional (post-transcriptional) mechanisms play a role in regulating apoB secretion.—**Leighton, J. K., J. Joyner, J. Zamarripa, M. Deines, and R. A. Davis.** Fasting decreases apolipoprotein B mRNA editing and the secretion of small molecular weight apoB by rat hepatocytes: evidence that the total amount of apoB secreted is regulated post-transcriptionally. *J. Lipid Res.* 1990. **31:** 1663-1668.

Supplementary key words apoB • mRNA processing • metabolic regulation • VLDL secretion

ApoB is essential for the assembly of triglyceride-rich lipoproteins (1). There are two molecular weight forms of apoB derived from a single gene (1-4). The larger molecular weight form (apoB_L) consists of 4563 amino acids, while the smaller molecular weight form (apoB_S) consists of the amino terminal 48% of the larger form (5-8). In humans, the large molecular weight form is secreted by the liver, whereas the small molecular weight form is secreted by the intestine (1). Similar to humans, rat intestine secretes only apoB_S, whereas unlike the human, rat liver secretes both apoB_L and apoB_S (1, 2, 9-13). The mechanism accounting for the different molecular weight forms of apoB involves editing of the initial RNA transcript (5-7). In the unedited RNA, a CAA (glutamine) appears at codon 2353. As a result of editing, nucleotide 6666 (C) becomes a U, forming a stop codon (UAA). The edited RNA codes for a truncated form of apoB. RNA editing has been shown to account for the selective synthesis of the small molecular weight form of apoB by both the intestine and rat liver (14).

Fasting has been shown to decrease the ratio of apoB_S:apoB_L in plasma and in lipoproteins secreted by rat liver (12). Studies from our laboratory indicate that the ratio of apoB_S:apoB_L can be influenced by dietary state; fasting decreases, whereas a carbohydrate-enriched diet increases this ratio (13, 15, 16). Furthermore, the ratio of apoB_S:apoB_L varies in parallel to: *1)* the rate of lipogenesis, *2)* the secretion of VLDL lipids, and *3)* the total

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; DEPC, diethyl pyrocarbonate; apoB_S, small apolipoprotein B; apoB_L, large apolipoprotein B; PCR, polymerase chain reaction.

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amount of apoB (apoB_S plus apoB_L) secreted (13, 15, 16). These data suggest that these parameters may be coordinately regulated. Similar parallel relationships are displayed in rat liver during fetal and postnatal development (17, 18). The recent finding that apoB RNA editing is sensitive to thyroid status (14) suggests that this process may be affected by hormones.

To gain an understanding of the regulation of apoB secretion, we examined the mechanism through which fasting selectively decreases the secretion of apoB_S. The results show that fasting decreases the relative amount of the edited RNA in the liver. Moreover, while the percent RNA having the stop codon was markedly decreased by fasting, there was no change in total amount of mRNA coding for apoB. Thus, while decreased RNA editing can account for the shift to a higher apoB_S:apoB_L ratio secreted by hepatocytes from fasted rats, post-transcriptional mechanisms regulate the total amount of apoB (apoB_S plus apoB_L) secreted.

EXPERIMENTAL PROCEDURES

Preparation and labeling of hepatocytes

Hepatocytes from male Sprague-Dawley rats (150–250 g) that were fed or fasted for 3 days were isolated and cultured as described in detail (19). Four hours after plating the cells in serum containing DMEM, the medium was replaced with 5 ml of methionine-free MEM with antibiotics, insulin, and [^{35}S]methionine (60 μCi/ml, sp act > 800 Ci/mmol). The cells were incubated for an additional 4 h. After this time, the media from two plates were combined and centrifuged and fractionated on a 0.5 m agarose column as described in detail (20).

Immunoprecipitation of eluted apolipoprotein B

Aliquots of each fraction were brought to 0.1% SDS and immunoprecipitated with a polyclonal rabbit antibody specific for apoB, as previously described (16). The immunoprecipitates were subjected to SDS/PAGE using a 1–20% linear acrylamide gradient. The proteins were excised from the gel and the amount of radioactivity in each gel slice was quantitated by scintillation counting.

Isolation of RNA

Total hepatic RNA from fed and fasted rats was isolated by the acid-guanidine-phenol-chloroform extraction method (21). Upon excision, 1 g of rat liver was added to 10 ml of GT solution (4 M guanidium isothiocyanate, 0.5% N-lauroyl sarcosine, 25 mM sodium citrate (pH 7), and 0.1 M β-mercaptoethanol) and minced with a tissue homogenizer. The solution was acidified, extracted with phenol-chloroform, and precipitated with isopropanol. The RNA pellet was dissolved in 5 ml of GT solution, precipitated with isopropanol, dissolved in sterile DEPC-treated water, and stored at −70°C until use.

Slot blot analysis of apoB mRNA

The relative abundance of apoB mRNA was quantitated using slot-blot analysis of total liver RNA, using beta-actin as the reference mRNA as described in detail (20). The cDNA probes used were: pRAT24, an 800 bp apoB cDNA 5' to nucleotide 6666 (obtained as a generous gift from James Scott), a 3.3 kbp apoB cDNA 3' to nucleotide 6666 (a generous gift from Aldons Lusis (22)), and actin pHFβA-1 (23).

Polymerase chain reaction amplification of apoB mRNA

One μg of total RNA from each liver was transcribed into single stranded cDNA in a 25-μl reaction containing 50 mM KCl, 20 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 40 units RNasin, 100 μg/ml BSA, 1 mM each dNTP, 10 mM DTT, 100 pmol random hexamer primers, and 14 units of AMV reverse transcriptase in a 0.5 ml siliconized microfuge tube. After a 1-h incubation at 42°C, the reaction mixtures were boiled for 5 min and centrifuged briefly. The polymerase chain reaction was performed using the GeneAmp kit from Perkin Elmer Cetus using manufacturer's protocols with the addition of BSA to a final concentration of 100 μg/ml and $MgCl_2$ to 2 mM. The primers used were 5'-TAGAGGATCCCTGAGCAGGCTT CCTCAGCAG (sense) and 5' TTTAAAGCTTCAATGA TTCTATCAATAATCTG (antisense) and corresponded to the rat apoB and oligonucleotide sequence of Tennyson et al. (24). These primers contain a BamH1 and a HindIII site at their ends. One μg of each primer was added to the PCR reaction. Each reaction was overlayered with 100 μl mineral oil and cycled 25 times for 3 min at 94°C, 2 min at 54°C, and 3 min at 74°C with a final extension at 74°C for 7 min using a Perkin Elmer Cetus DNA Thermal Cycler. After amplification the reactions were extracted with phenol-chloroform and precipitated with ethanol. The PCR products were digested with BamH1 and HindIII, subjected to electrophoresis on a 2% agarose gel, and the 297 bp PCR-amplified DNA fragment was extracted and purified from agarose with GeneClean (Bio 101). These fragments were then ligated into BamH1-HindIII cut, gel-purified pUC 19 and the resultant plasmid DNA with the apoB insert was used to transform *E. coli* Dh5α to ampicillin resistance.

Sequence analysis of PCR apoB clones

Plasmid DNA was isolated from 1.5-ml overnight cultures by brief centrifugation to pellet the bacteria and dissolved in 200 μl of sucrose, Triton X-100, EDTA, Tris (STET) buffer (25). After a 5-min incubation with 0.5 mg/ml lysozyme, the suspension was boiled for 40 sec and centrifuged for 15 min at room temperature. The supernatant was transferred to a new microfuge tube and precipitated with an equal volume of isopropanol. The

plasmid DNA was pelleted by centrifugation, dissolved in distilled water, precipitated with ethanol, washed, and dried under vacuum. The pellet was resuspended in 100 μl of 10 mM Tris–0.1 mM EDTA (pH 8.0) and digested with 100 μg/ml heat-treated RNase A for 30 min at 37°C. The resulting DNA was phenol-extracted, purified using GeneClean, and denatured according to manufacturer's protocols (United States Biochemical Corporation) to allow for the annealing of the sequencing primer. The DNA was sequenced using the Sequenase kit (United States Biochemical Corporation) and 20 ng of a gel-purified apoB sense primer (ATCTGACTGGGAGAGA CAAGTAG). Sequence reactions were analyzed by electrophoresis on 5% acrylamide–8 M urea denaturing gels followed by autoradiography.

Statistical analysis

All data are presented as the mean ± SD. Statistical differences were determined using Student's *t* test (double-tailed *P* values).

RESULTS

Previous studies have shown that fasting selectively decreases the secretion of $apoB_S$ (16). We determined the lipoprotein form in which apoB is secreted by control and fasted cells. After labeling cells with [^{35}S]methionine for 4 h, the medium was concentrated and separated using agarose chromatography (20). Previous results show that the elution of triglycerides (VLDL) is complete by fraction 23 (20). Consistent with previous results (14), fasting selectively decreased the secretion of $apoB_S$, whereas the secretion of $apoB_L$ was unchanged (**Fig. 1**). While essentially all of the $apoB_L$ was found secreted with triglyceride (i.e., eluted by fraction 23), approximately 15% of $apoB_S$ was found in fractions that contained no triglyceride (Fig. 1). A portion of apoB has been shown to be secreted by perfused rat liver as a component of HDL (26). The finding that a portion of $apoB_S$ is secreted without triglyceride may explain kinetic data suggesting the existence of more than one secretory pathway for this form of apoB (27). The major change in $apoB_S$ secretion caused by fasting was decreased in the proportion of $apoB_S$ secreted with triglyceride fractions 19–23. The increased proportion of $apoB_S$ secreted without triglyceride displayed by cells from fasted rats is probably caused by markedly decreased availability of triglyceride (16). We examined whether decreased apoB mRNA could account for the decreased secretion of $apoB_S$.

Relative abundance of apoB mRNA

Total hepatic RNA, obtained from groups of control and fasted rats, showed no difference in the yield of RNA per gram of liver. The RNA was intact as shown by Northern analysis which produced a single 14.2 kb band, detected using a 3.3 kbp cDNA clone corresponding to the 3' end of the apoB mRNA (data not shown). The finding of a single mRNA for apoB in the rat is consistent with previous data showing that in this species there is a single polyadenylation signal (28). ApoB cDNAs containing either the 5' or 3' coding sequences (relative to the stop codon) were both used to quantitate mRNA (see legend Fig. 2). Using both 5' or 3' probes, it was found that fasting did not significantly alter the relative abundance of apoB mRNA (**Fig. 2**). These data suggest that fasting decreases total apoB ($apoB_S$ plus $apoB_L$) secretion without changing the abundance of apoB mRNA.

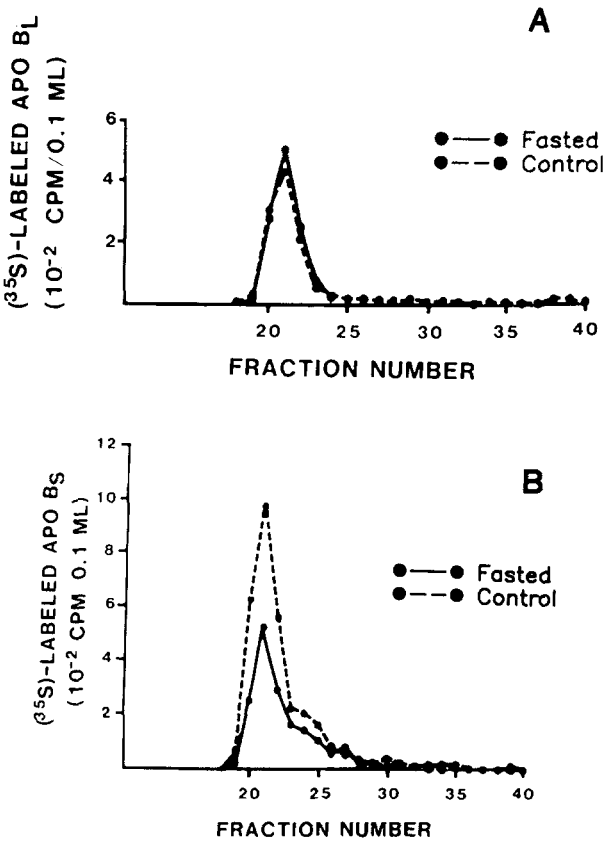

Fig. 1. Elution of ^{35}S-labeled apoB. Hepatocytes from control and fasted rats were plated as described. After 4 h, the media were changed to serum-free, methionine-free, modified MEM containing 300 μCi of [^{35}S]methionine/100-mm culture dish. After 4 h, the media from two plates were combined and fractionated with the 0.5 m agarose column. Fifty-μl aliquots of fractions 19–40 were incubated with anti-apoB antisera and precipitated with protein A-Sepharose. The precipitated proteins were separated with SDS-polyacrylamide gel electrophoresis, and the labeled $apoB_L$ (panel A) and $apoB_S$ (panel B) were quantitated by scintillation counting. This experiment has been performed a total of three different times (three individual hepatocyte preparations in each group). The results were similar in all three experiments. Using rat serum lipoprotein standards, VLDL eluted in fractions 19–23 and HDL eluted in fractions 23–28 (see reference 20).

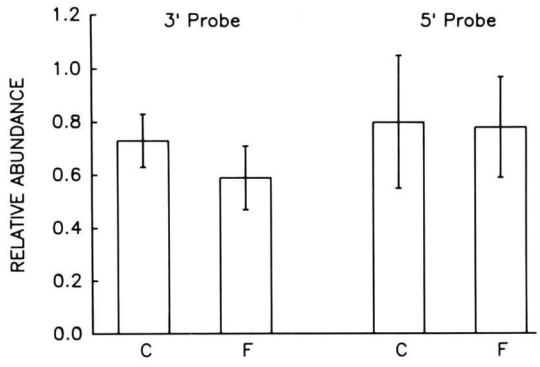

Fig. 2. Slot blot analysis of apoB RNA. Total hepatic RNA from control (C) and 3-day fasted (F) rats was applied to nitrocellulose strips in amounts from 0.5 to 5.0 μg. The filters were hybridized with two nick-translated ^{32}P-labeled apoB cDNA probes: *1*) a 3.3 kbp probe (3' probe) and *2*) an 800 bp probe (5') relative to nucleotide 6666 of the apoB mRNA. After autoradiography, the films were scanned with a video densitometer and the slope of the line of absorbance versus concentration was determined. The relative abundance of apoB RNA was determined as the ratio of the slope of apoB:β-actin, a constitutive reference probe. Results are expressed as mean ± SD for six control and four fasted rat livers.

Effect of fasting on the proportion of mRNA containing the stop codon (UAA)

We determine whether fasting decreases the secretion of apoB$_S$ by decreasing the amount of edited apoB RNA (coding for the stop codon). Hepatic mRNA was isolated from control and fasted rats, amplified using the polymerase chain reaction, cloned into pUC 19, and sequenced. A minimum of 40 independent cDNA clones were obtained and sequenced for each individual rat (n = 3 rats in each group). The sequencing approach is shown in **Fig. 3**. For each sequencing gel, a standard DNA was sequenced allowing identification of nucleotide 6666 (at the C of the CAA codon Fig. 3, left side). Using dideoxythymidine triphosphate, the presence or absence of the T (corresponding to the U of the RNA stop codon) was determined.

Each individual pUC19 isolate was sequenced and the proportion of either C (gln) or U (stop) in 40 separate mini-preps was determined for each rat liver. RNA obtained from the fasted rats showed a marked 60% reduction in the proportion having a UAA (stop codon) (**Fig. 4**). These data show for the first time that fasting, which selectively decreases apoB$_S$ secretion by 50%, is associated with a similar 60% decrease in the proportion of apoB mRNA coding for apoB$_S$ (i.e., having the UAA stop codon). However, since total apoB mRNA is not affected by fasting (Fig. 2), the total amount coding for apoB$_L$ is increased by 3.9-fold. Clearly, fasting does not increase apoB$_L$ secretion (Fig. 1). These data show that while RNA editing correlates with the apoB$_S$:apoB$_L$ ratio, the relative abundance of total apoB mRNA does not dictate the amount of total apoB that is secreted by hepatocytes.

DISCUSSION

Previous studies show that fasting affects the secretion of individual apolipoproteins differently; apoB$_S$ is reduced, apoB$_L$ is unchanged, and apoE is increased (16). These data suggest that the secretion of individual apolipoproteins is regulated independently (16). Additional studies show that the increased secretion of apoE was associated with an enrichment in the relative abundance of apoE mRNA (20), suggesting that augmented synthesis was responsible. The major goal of this study was to define the mechanism(s) responsible for the selective impairment of apoB$_S$ secretion. The results show that the relative abundance of total apoB mRNA does not change (Fig. 2), whereas the proportion of mRNA coding for apoB$_S$ is decreased by 60% (Fig. 4). The data showing that fasting decreases both the amount of apoB$_S$ mRNA and the secretion of apoB$_S$ to the same extent (50 to 60%) indicates that decreased editing (formation of the stop codon) is the mechanism responsible. To our knowledge, this is the first demonstration that apoB mRNA editing in rat liver is varied in response to dietary state.

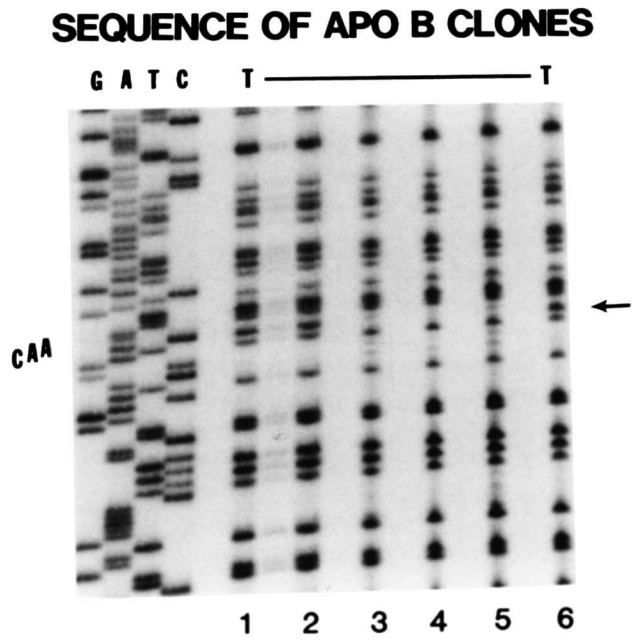

Fig. 3. Sequence analysis of apoB cDNA clones. ApoB RNA was amplified using the polymerase chain reaction and cloned into pUC19. Over 40 independent bacterial clones were obtained for each of three control and three fasted rat livers. The sequence of one of these clones is shown on the left. The CAA identifies the site of the glutamine codon in apoB$_L$. The six representative clones to the right were sequenced using dideoxythymidine to determine the presence (lanes 1, 2, and 6) or the absence (lanes 3–5) of a thymidine residue in place of the cytidine of the glutamine codon. The arrow on the right indicates the position of the nucleotide of interest.

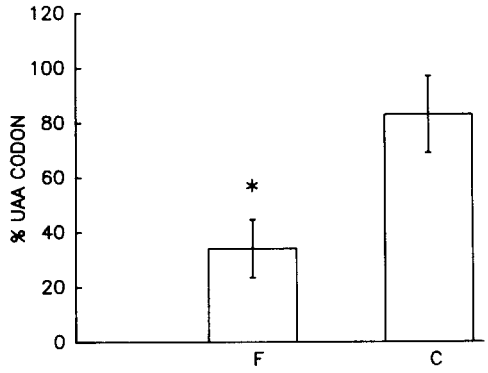

Fig. 4. Effect of fasting on the presence of the stop codon in hepatic apoB RNA. ApoB cDNA clones were sequenced and analyzed as described in Fig. 3, and the % clones containing the stop codon (UAA) were determined. Values presented are mean ± SD (n = 3);* denotes significant difference at $P<0.01$.

The combined data show a discordance between the amount of total apoB secreted (decreased by fasting) and the relative abundance of apoB mRNA (unchanged by fasting). This finding is consistent with those of others that show no change in apoB mRNA levels in livers and cells displaying significant changes in the secretion of apoB (29–31). The secretion of apoB by HepG2 cells varies in response to hormones and lipids, whereas the relative abundance of apoB mRNA does not change (29). Similarly, thyroid hormone has a dramatic impact on the rate of secretion of apoB, without similar changes in the relative abundance of apoB mRNA (30). Furthermore, eicosapentaenoic and docosahexaenoic acids impair the secretion of apoB by HepG2 cells without changing the relative abundance of apoB mRNA (31). It appears that hepatic content of apoB RNA is not solely responsible for regulating apoB secretion.

Rat liver responds to several different conditions: fasting (16), carbohydrate feeding (15), development from neonate to adult (18), and thyroid hormone (30) by changing, in parallel, lipogenesis, triglyceride secretion, and apoB secretion and the ratio of secreted $apoB_S$:$apoB_L$, suggesting that a common regulatory factor may be responsible. This factor may afford a mechanism by which the synthesis of the essential components involved in VLDL assembly is coordinately regulated. A *trans*-acting regulatory protein (C/EBP) has been described that coordinately regulates the synthesis of several lipogenic enzymes in liver, adipose tissue, and intestine (32). It is possible that a *trans*-acting protein or a protein having an ability to coordinately regulate the expression of genes expressing the processes involved in VLDL assembly may exist.

Since $apoB_S$ and $apoB_L$ are each capable of assembling a VLDL particle, in regard to VLDL assembly no advantage is apparent for varying the editing of apoB mRNA in parallel to lipogenic state. However, VLDL that are enriched with $apoB_S$ are cleared from plasma at a rate greater than those enriched in $apoB_L$ (11, 33–35). The switch to VLDL enriched in $apoB_S$, having a more rapid clearance from plasma, with increased triglyceride synthesis and secretion, may provide a mechanism that limits the accumulation of VLDL in the plasma of rats. The inability of human liver to edit apoB mRNA may, in part, explain why humans show a greater increase in plasma lipoprotein levels in response to dietary fat than do rats.

The combined data show that hepatic apoB mRNA levels are not the sole determinant of apoB secretion. With the proviso that apoB mRNA levels reflect apoB synthesis, a post-translational mechanism may determine apoB secretion. Intracellular degradation of de novo synthesized apoB has been demonstrated in cultured rat hepatocytes (27). Additional studies show that this degradation is likely to occur in a compartment of the endoplasmic reticulum (36). Furthermore, apoB is not quantitatively translocated across the endoplasmic reticulum membrane, resulting in two functionally distinct pools (37). The translocated pool enters the VLDL assembly pathway. In contrast, the non-translocated pool does not enter the secretory pathway and is likely to be degraded. If sorting of apoB into different pathways is sensitive to metabolic regulation, intracellular degradation may explain how fasting decreases apoB secretion independent of RNA levels.

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