Insulin promotes the biosynthesis and secretion of apolipoprotein B-48 by altering apolipoprotein B mRNA editing

(hyperinsulinemia/hypertriglyceridemia/very low density lipoprotein)

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ABSTRACT Long-term insulin treatment selectively stimulates secretion of the truncated form of apolipoprotein B (apoB), apoB-48, from primary rat hepatocytes in culture. Chronic treatment with insulin at 400 ng/ml causes a 3-fold increase in total apoB secretion, with apoB-48 making up about 75% of that increase. apoB-48 is the protein product generated by translation of full-length apoB mRNA which has been modified by a posttranscriptional editing mechanism. Editing changes codon 2153 in the middle of the apoB-100 coding region from CAA, coding for glutamine, to UAA, a translation stop signal. We therefore examined the effect of insulin treatment on the ratio of edited to nonedited apoB mRNA in RNA isolated from primary rat hepatocyte cultures. There was a dramatic shift in the ratio of edited versus nonedited forms of apoB mRNA, from about 1:1 in untreated cells to 7:1 in insulin-treated cells. Insulin exerted a dose-dependent effect on apoB secretion and apoB mRNA editing over the range of insulin concentrations studied (0.4–400 ng/ml). In contrast, oleic acid, which also increased apoB (B-48 and B-100) secretion, had no significant effect on the ratio of apoB-48 to apoB-100 particles secreted and no effect on the proportion of edited apoB mRNA. Neither insulin nor oleic acid affects total apoB mRNA levels as assayed by Northern blot analysis. These data strongly suggest that insulin stimulates biosynthesis and secretion of apoB-48 in rat hepatocytes by regulating the proportion of edited apoB mRNA.

Apolipoprotein B (apoB) is an essential structural component of triglyceride-rich very low density lipoproteins (VLDLs) and chylomicrons. The VLDLs are the metabolic precursors of the low density lipoprotein (LDL), the main carrier of cholesterol in humans. apoB is one of the ligands involved in clearance of LDL through binding to the LDL receptor; thus apoB is central to cholesterol homeostasis in the circulation. apoB is also essential for triglyceride (TG) transport to peripheral tissues (1).

There are two forms of apoB, which are coded for by a single gene. The full-length translation product is apoB-100; it is a 512,000-Da polypeptide synthesized in the liver and secreted as an obligatory component of VLDL. The shorter form, apoB-48, is a 250,000-Da polypeptide identical to the amino-terminal 48% of apoB-100 and synthesized in the intestine as an integral part of the chylomicrons. In the rat, apoB-48 is also synthesized in the liver and secreted in VLDL. The mechanism for generating these two forms of apoB is a post-transcriptional RNA modification in which the CAA codon for glutamine-2153 in apoB-100 is changed to UAA, resulting in an in-frame translation stop signal (2). The polypeptide translation product of this edited RNA is apoB-48.

As part of studies to define the role of insulin in the pathogenesis of dyslipidemia in the hyperinsulinemic state, we examined the effects of long-term (5-day) insulin exposure on apoB secretion from primary cultures of rat hepatocytes. Chronic exposure to insulin selectively stimulates the secretion of apoB-48 both in VLDL (M.B.E., unpublished data) and in total apoB. These results indicated that insulin might modulate the apoB mRNA editing mechanism, and we examined the effect of insulin on total apoB mRNA levels and on the ratio of edited to nonedited apoB mRNA. Our results demonstrate that insulin is a primary modulator of apoB editing and that insulin stimulates apoB-48 secretion from rat hepatocytes by increasing apoB mRNA editing.

METHODS

Primary Hepatocyte Culture. Adult male Sprague–Dawley rats were obtained from Harlan Laboratories, and fed ad lib with rat chow (no. 5001; Ralston Purina). Hepatocyte suspensions were prepared by using type 2 collagenase (lot 67158M; Worthington) and dispersion techniques as described previously (3), modified for the preparation of monolayer hepatocyte culture (4). Cells were resuspended at a density of approximately 1.0 × 10⁶ cells per ml in Dulbecco’s modified Eagle’s medium supplemented with 20% (vol/vol) fetal bovine serum, and 3 ml was plated in 60-mm dishes coated with rat tail collagen (Collaborative Biochemical Products, Bedford, MA). At 4 hr, the plating medium and nonadherent cells were aspirated and replaced with feeding medium (3 ml per plate). Feeding medium consisted of William’s medium E (formulation 79-5204; GIBCO/BRL) supplemented with 0.64 mM L-ornithine, 38 mM sodium bicarbonate, 10 mM Hepes, 10 mM dextrose, streptomycin at 100 μg/ml, penicillin G at 100 international units/ml, gentamicin at 50 μg/ml, and amphotericin B at 2.5 μg/ml. The feeding medium was further supplemented with 100 nM dexamethasone, 50 nM triiodothyronine, bovine serum albumin at 1 mg/ml, linoleic acid at 5 μg/ml, and trace minerals (0.1 μM CuSO₄, 3 nM NaSeO₃, and 50 pM ZnSO₄) as described by Enat et al. (5). Bovine insulin (Sigma) was added to the feeding medium to produce concentrations of 0–400 ng/ml (0–67 nM), according to the treatment protocol. Medium was aspirated and replaced with fresh medium daily. Unless specified otherwise, pulse labeling experiments were carried out with the corresponding feeding medium.

Abbreviations: apoB, apolipoprotein B; apoB-100, full length apoB protein; apoB-48, amino-terminal 48% of apoB-100; LDL, low density lipoprotein; TG, triglyceride; VLDL, very low density lipoprotein.

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Measurement of Secreted ApoB-48 and ApoB-100. Following 5 days of treatment with various concentrations of insulin, the cells were washed once with Dulbecco’s modified Eagle’s medium with 1-methionine (GIBCO/BRL), pulsed for 30 min with [35S]methionine (Tran35S-label; ICN) in methionine-free medium at 200 μCi/ml (1 Ci = 37 GBq), and chased for 6 hr in the appropriate treatment medium with or without 1 mM oleic acid (as a complex with bovine serum albumin at 15 mg/ml).

The conditioned medium was collected, and the cells were rinsed with 0.5 ml of ice-cold phosphate-buffered saline and the rinses were added to the medium. The apoB was immunoprecipitated from the medium by incubating with the appropriate anti-apoB antibody. The conditioned medium was cleared with normal rabbit serum (Sigma) and Pansorbin (Calbiochem), diluted 1:1 in the described immunoprecipitation buffer, and incubated for 18 hr at 4°C, and immune complexes were precipitated with Pansorbin. The immune complexes were washed three times and the apoB-48 and apoB-100 were separated by SDS/PAGE. The apoB bands were visualized by autoradiography, and the bands were cut out, dissolved, and quantitated by liquid scintillation spectrometry. Trichloroacetic acid-precipitable 35S-labeled material was measured as an index of overall protein synthesis. Total cell protein was measured with the BCA protein assay system (Pierce).

Northern Blot Analysis of Total apoB mRNA. The probe used to detect the apoB mRNA was a 297-bp DNA fragment homologous to the sequence from 205 bases upstream to 91 bases downstream from the edited cytosine in glutamine codon 2153 (7). The PCR product resulting from one of the PCR amplifications described below was digested with HindIII and BamHI. The probe was generated by subcloning the resultant 297-bp fragment in pTZ18R (United States Biochemical). The probe (50 ng) was labeled with [α-32P]dCTP by using a random primers labeling kit.

Total RNA was isolated from hepatocyte monolayers by overlaying the cells with 1.5 ml of RNAzol B (Biotex Laboratories, Houston), following the manufacturer’s protocol. Ten micrograms of total RNA was loaded per lane of a formaldehyde/0.8% agarose gel and blotted onto nitrocellulose; duplicate samples were electrophoresed and stained with ethidium bromide to assess the integrity of the RNA. The filters were vacuum baked, and hybridization was carried out according to published protocols (8). Specifically hybridized apoB transcripts were visualized by autoradiography using Kodak X-Omat AR film.

PCR Amplification of apoB mRNA. All of the oligonucleotide probes and primers used in this work were synthesized on an Applied Biosystems 381A automated DNA synthesizer and purified by 7 M urea/12% PAGE. cDNA was synthesized from 1 μg of total cellular RNA by reverse transcriptase using random primers, with reagents and protocol from the cDNA Cycle Kit (Invitrogen). The cDNA was amplified by PCR as described (9), using 0.46 μM each of a sense and anti-sense primer for rat apoB developed by Tennyson et al. (7) and reaction components and Taq DNA polymerase from Perkin-Elmer. The 304-bp PCR products were purified by using GeneClean (Bio 101).

Primer Extension Analysis. Primer extension analysis was carried out by using a 27-base oligonucleotide primer complementary to the region starting 8 bases downstream from the edited site in rat apoB mRNA, as described by Wu et al. (10). The primer was labeled by using a 5'-end-labeling kit and protocol (Promega) and 30 μCi of [α-32P]ATP (3000 Ci/ mmol). Half of the product from each PCR was assayed by denaturing the DNA at 95°C for 5 min, then annealing with an ∼5-fold molar excess of labeled primer in 34 mM Tris-HCl, pH 8.3/50 mM NaCl/5 mM MgCl2/5 mM dithiothreitol at 58°C for 45 min. The extension was carried out at 42°C for 30 min after the addition of 0.5 mM each dATP, dTTP, dCTP, and 2',3'-dideoxy-GTP and 5 units of avian myeloblastosis virus reverse transcriptase according to Driscoll et al. (11). The reaction products were precipitated with ethanol, electrophoresed on a 12% polyacrylamide sequencing gel, and analyzed by autoradiography with Kodak X-Omat AR film. The bands corresponding to edited (41-mer) or nonedited (36-mer) products were cut out of the gel and quantitated by liquid scintillation spectrometry.

RESULTS

When rat hepatocytes in culture are chronically exposed to physiologic levels of insulin, modeling the hyperinsulinemia common to insulin-resistant states in humans, there is a specific stimulation of the secretion of total apoB from the hepatocytes. This increase in immunoprecipitable medium apoB can be accounted for mainly by an increase in apoB-48 secretion. Following exposure of primary cultures of rat hepatocytes to insulin (4–400 ng/ml; 0.67–67 nM) for 5 days, we observed a dose-dependent increase in the rate of total apoB secretion, as shown in Fig. 1 A. The effect of insulin on apoB secretion is specific, being 4-fold greater than the
generalized stimulation of protein synthesis after insulin treatment as measured by trichloroacetic acid-precipitable incorporated $^{35}$S)methionine (F.E.T., unpublished data). The percent increase in secretion (relative to no insulin treatment and normalized for total cell protein) for apoB-48 was 367% after treatment with insulin at 4 ng/ml and 1003% after treatment with insulin at 400 ng/ml. For apoB-100 the percent increases in secretion were 240% and 290%. Although apoB-100 secretion increases slightly following insulin treatment, the predominant effect of insulin is on apoB-48, the product of the edited apoB mRNA. This is reflected by an increase in both the absolute amount of apoB-48 secreted (Fig. 1A) and the percentage of apoB-48 relative to total apoB secreted. apoB-48 is 65–70% of the apoB secreted after treatment without insulin and about 90% of the apoB secreted after treatment with insulin at 400 ng/ml (Fig. 1B). Insulin increased apoB-48 secretion both in the presence and absence of added fatty acid (Fig. 1B).

Insulin resistance in the intact animal is associated with high concentrations of plasma free fatty acid in addition to hyperinsulinemia (12). Free fatty acid independently stimulates TG synthesis and VLDL secretion in perfused liver (13) and in hepatocytes (14). We therefore decided to determine if stimulation of apoB secretion by insulin was independent of, or related to, the presence of free fatty acid. The addition of 1 mM oleic acid to the chase medium increased the absolute output of apoB in the absence of insulin and amplified the effect of insulin on apoB secretion at low (4 ng/ml), but not at high (400 ng/ml), insulin concentrations (Fig. 2). Conversely, insulin provides a potent stimulus to apoB secretion in the absence of olate, with the maximum effect on apoB secretion being equal to or greater than that observed in the presence of oleic acid. Furthermore, the addition of fatty acid had no effect on the distribution between apoB-48 and apoB-100 (Fig. 1B). Thus, although the high free fatty acid levels that accompany hyperinsulinemia in vivo probably contribute to the observed hypertriglyceridemia, insulin is a potent stimulus to VLDL-apoB secretion independent of fatty acid.

Conceivably, the observed enhancement in the rate of secretion of apoB could be mediated by transcriptional mechanisms. To determine if insulin altered the steady-state levels of cellular apoB (edited and nonedited) mRNA, total RNA isolated from the treated hepatocytes was analyzed by Northern blot hybridization to a cDNA probe which recognizes both the edited and unedited versions of apoB mRNA. There was no measurable change in the amount of apoB mRNA from hepatocytes treated with various amounts of insulin; the addition of 1 mM oleic acid to the medium was similarly inconsequential in regulating the steady-state levels of apoB mRNA (Fig. 3). Therefore, neither insulin nor fatty acid appears to stimulate apoB biosynthesis and secretion by mechanisms which regulate steady-state levels of mRNA.

Our results showed a dramatic increase in apoB-48 protein secretion after insulin treatment, both in absolute amount and relative to apoB-100. The absence of an effect of insulin on total apoB mRNA indicated that the effect of insulin on the proportion of apoB-48 might be mediated through regulation of the amount of edited mRNA. To study the effects of insulin on the ratio of edited apoB mRNA, we isolated total RNA and synthesized cDNA to amplify a 297-base region of the apoB mRNA (including the editing site) by PCR. The resulting mixture of "edited" (TAA) and "nonedited" (CAA) PCR products was analyzed by primer extension to quantitate the amount of each species present after various insulin treatment conditions. This assay for examining the ratio of edited to nonedited apoB mRNA has been previously shown to be meaningful over a wide range of editing ratios (10), and all of our data fall within this range. An autoradiograph depicting the PCR products, indicative of the edited and unedited forms of apoB mRNA, from hepatocytes treated with different concentrations of insulin is shown in Fig. 4A. This representative experiment illustrates that insulin treatment increases the ratio of edited to unedited apoB mRNA in a dose-dependent manner. In contrast, Fig. 4B demonstrates that cultivation of hepatocytes in medium supplemented with 1 mM oleic acid for the final 6 hr of culture has no effect on apoB mRNA editing. Oleic acid is ineffective regardless of whether it is presented alone or in combination with insulin, and it does not alter the effect of insulin. Oleic acid was equally ineffective when it was included for the entire 5 days of culture (F.E.T., unpublished data). The radioactive bands visualized by autoradiography were cut out, and incorporation of $^{32}$P into the bands corresponding to edited or nonedited apoB mRNA was measured by liquid scintillation spectrometry. The percentage of edited message was calculated for each reaction, and the results are shown in Table 1. Insulin treatment has a clear dose-dependent effect on the

![Fig. 2. Effect of fatty acid on insulin-modulated secretion of total apoB. The experimental conditions are the same as described in the legend of Fig. 1. The absolute amount of apoB-48 and apoB-100 secreted after treatment with insulin at 0, 4, or 400 ng/ml with or without 1 mM oleic acid, normalized against total cell protein, is presented.](image)

![Fig. 3. Northern blot analysis of total apoB mRNA. Cells were harvested on the fifth day, 6 hr after exposure to fresh feeding medium prepared with or without 1 mM oleic acid. Total RNA was isolated from hepatocytes treated with insulin at the indicated concentrations. Duplicate samples of 10 μg of total RNA were run on an 0.8% agarose/formaldehyde gel. Half of the gel was blotted onto nitrocellulose and hybridized to a $^{32}$P-labeled apoB cDNA probe. The autoradiograph shows that the levels of the 14-kb apoB mRNA remain unaltered regardless of the treatment with insulin (0, 4, and 400 ng/ml) with or without fatty acid. Below it is a photograph of duplicate lanes from the gel stained with ethidium bromide. The 18S and 28S ribosomal RNA bands serve as a control, demonstrating the integrity of the RNA and loading of equal amounts of RNA in each lane.](image)
percentage of edited apoB, and this effect of insulin is not modified by the addition of fatty acid.

On the basis of these observations, we conclude that apoB biosynthesis and secretion in primary hepatocytes in culture are subject to regulation by insulin treatment. Furthermore, our experimental findings strongly suggest that the primary site of insulin action on apoB biosynthesis in this system is the modulation of the molecular mechanisms of posttranscriptional editing of apoB mRNA.

DISCUSSION

It has been difficult to define the role of insulin in the development of the hypertriglyceridemia which often accompanies non-insulin-dependent diabetes and hyperinsulinemia. Increased hepatic secretion of VLDL-TG and apoB occurs in vivo in hyperinsulinemic rats. The VLDL overproduction occurs whether hyperinsulinemia results from sucrose feed-

Table 1. Percentage of edited apoB message at various insulin concentrations, with or without fatty acid

<table>
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<tr>
<th>Fatty acid</th>
<th>% edited apoB message after culture in insulin</th>
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<tr>
<td></td>
<td>0 ng/ml</td>
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<tr>
<td>Oleate (1 mM)</td>
<td>69.7 ± 6.3</td>
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<tr>
<td>None</td>
<td>67.2 ± 4.1</td>
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*These percentages are from a single determination; all of the other percentages are from three or four independent hepatocyte preparations. The values presented are the mean ± SEM.

ing (15) or in genetic models of insulin resistance such as the SHR/N-cp rat (16) or the ICR:LA corpulent rat (17). However, the direct role of insulin, if any, in the VLDL overproduc-
tion observed with insulin resistance has not been de-
dined. There are conflicting reports on the effect of insulin on hepatic secretion of apoB. Pullinger et al. (18) and Dashti et al. (19) studied apoB secretion and apoB mRNA levels in the human hepatoma cell line HepG2. Both groups observed a decrease in apoB secretion upon short-term (<24-hr) exposure of cells to insulin, with no change seen in cellular apoB mRNA levels. They observed a decrease in apoB secretion after insulin treatment, in spite of an increase in cellular TG. Similar results were observed in primary rat hepatocyte culture (20, 21). In contrast, Bartlett and Gibbons (22) demonstrated that chronic (>24-hr) insulin exposure of rat hepatocytes stimulated VLDL secretion.

These observations led us to examine the effects of long-
term (5 day) insulin exposure on apoB secretion from primary rat hepatocyte cultures. We clearly show that long-term treatment with physiologic concentrations of insulin stimulates secretion of apoB. Although the acute inhibitory effects of insulin may be important in short-term (i.e., the immediate postprandial state) regulation of VLDL secretion, our long-term insulin treatment model more closely mimics the in vivo situation in hyperinsulinemia.

In the intact animal VLDL secretion is regulated by the interplay between a complex set of nutritional and hormonal factors, making it difficult to determine which factors are causal and which are simply linked effects. Insulin resistance is accompanied by elevated plasma free fatty acid concentrations and hyperglycemia, as well as hyperinsulinemia, any of which may increase VLDL secretion (12, 23). In the present work we examined the effects of high levels of free fatty acid, which often accompany hyperinsulinemia. Oleic acid stimulates apoB secretion from hepatocytes, but insulin stimulation of apoB secretion is maintained in the presence of oleate. The presence of 1 mM oleate causes a shift in the insulin dose–response curve, leading to a plateau in apoB secretion at a lower insulin concentration (Fig. 2). This shift indicates insulin and oleic acid may be acting independently through a common mechanism to stimulate apoB secretion. Exposure of hepatocytes to either oleic acid or chronic insulin causes a marked increase in the secretion of total apoB. In contrast, previous work in rat hepatocytes demonstrated that short-term exposure to insulin leads to enhanced intracellular degradation and decreased secretion of apoB, particularly apoB-100 (21). Neither insulin nor fatty acid affects total apoB mRNA levels (Fig. 3), so a posttranscriptional mode of regulation is implicated. One of the major sites of regulation of apoB secretion appears to be the endoplasmic reticulum, where newly synthesized apoB is either appropriately lipidated to form a nascent VLDL particle or is degraded. In the case of oleic acid, increased availability of TG for VLDL formation reduces intracellular degradation of newly synthesized apoB (24). There is an increase in cellular TG after chronic insulin treatment (M.B.E., unpublished data), and the increased TG may reduce the degradation of apoB by the same mechanism observed for oleic acid. Although the increase in total apoB secretion, particularly the increase in apoB-100 secretion, observed after insulin treat-
ment may be the result of decreased intracellular degradation of apoB, the dramatic shift to secretion of apoB-48 (Fig. 1B) indicates that insulin has an additional site of action for regulating apoB secretion. Oleate treatment has no additional effect on the ratio of apoB-48 to apoB-100 secreted (Fig. 1B). These observations led us to examine the effects of insulin on the ratio of edited to nonedited apoB mRNA, and we observed a marked shift to the edited form of apoB mRNA in insulin-treated cells.
As noted by Seishima et al. (25) and Sjoberg et al. (26), the ratio of apoB-48 to apoB-100 secreted by the cells closely follows the fraction of apoB mRNA edited. Our results with insulin show the same relationship; the percentage of apoB mRNA edited goes from 67% with no insulin treatment to about 90% with insulin at 400 ng/ml (Table 1). Almost identically, the percentage of apoB-48 protein secreted goes from about 67% to 90% under the same conditions (Fig. 1B), indicating that editing is the major determinant of the form of apoB secreted.

A limited number of modulators affect the ratio of edited to nonedited RNA in vivo; these include thyroid hormone (27), growth hormone (26), fasting and refeeding (6, 9), developmental stage (28), and estrogen (25, 29). apoB mRNA editing tends to increase under conditions which increase cellular TG content and secretion and/or apoB secretion, such as when fasted rats are fed a high-carbohydrate diet (6). Conversely, the proportion of apoB-48 mRNA tends to decrease in conditions where VLDL apoB and TG secretion are diminished, such as fasting (6, 9). Unexpectedly, the ratio of edited mRNA decreases with estrogen treatment despite increased accumulation of intracellular TG and increased TG secretion (25, 29, 30). Thus, although there appears to be a correlation between lipogenesis and apoB mRNA editing levels, the relationship is not a simple one and may be uncoupled under a variety of physiological and/or pathophysiological conditions. The regulation of apoB mRNA editing is complex and depends on the interplay of metabolic and hormonal modulators. Our results indicate that insulin may be one of the primary modulators of apoB mRNA editing; in fact, the resultant changes in in vivo insulin levels may mediate the changes in editing observed in fasted, fasted-refed, and high-carbohydrate-fed animals.

It is important to note that, in contrast to the rat liver, which edits apoB mRNA, human liver has low levels of editing of apoB mRNA, as demonstrated by studies to date (28). Therefore, it is difficult to predict the effect of hyperinsulinemia on apoB secretion and apoB mRNA editing in human liver.

Insulin may regulate the editing mechanism through several possible mechanisms. Insulin may increase either the amount or activity of the recently characterized editing enzyme (31). Alternatively, insulin may act by increasing the amount or binding of another factor in the proposed "editing" complex, such as the 66-kDa liver protein that associates with the apoB RNA editing region (32). The effects of long-term insulin treatment on the editing enzyme and on the proteins that bind specifically to the editing site remain to be determined. The time course of insulin action in our culture system can be analyzed to determine whether the increase in apoB mRNA editing is an acute effect or occurs concurrently with the chronic insulin effect of increased total apoB secretion.

Long-term exposure of primary hepatocytes to insulin is a useful model for studying the effects of hyperinsulinemia on secretion of TG-rich lipoproteins by the liver. Understanding the role of insulin in the development of the hyperlipidemia observed in hyperinsulinemic states is essential. In this regard, it is significant that the present work demonstrates that insulin is an important modulator of the biogenesis and secretion by the liver of apoB, the primary apoprotein of TG-rich lipoproteins.

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