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Insulin modulation of newly synthesized apolipoproteins B-100 and B-48 in human fetal intestine: Gene expression and mRNA editing are not involved

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Abstract We investigated insulin's effect on intestinal lipid transport and, particularly, the biogenesis of apolipoproteins crucial to lipoprotein secretion. Adding insulin (3 mU) to the serum-free medium of cultured jejunal explants from human fetuses (17–20 weeks) reduced triglyceride and chylomicron production and inhibited apo B-48 and apo B-100 secretion. When apo B mRNA was assayed by RT-PCR and its editing by primer extension, no change was detectable following the addition of insulin. HDL lipid content, apo A-1 synthesis and RNA level were unaffected by insulin. Collectively, these results suggest that the insulin-stimulated decline in intestinal chylomicron output may involve apo B co- or post-translational modifications.

Key words: Fetus; Intestine; Apo B; Apo A-I; Chylomicron; mRNA editing

1. Introduction

Among the most important functions of the intestine is the transport of dietary fat in the form of lipoproteins [1]. The enterocyte is the unique site for chylomicron formation, but is also a source for very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) production [2,3]. These nascent lipoproteins contain apoproteins (apo) A-I and B as the major protein constituents [1–4].

The synthesis and secretion of intestinal and hepatic lipoproteins are exquisitely regulated by several factors, including hormones, growth factors and nutrients. Much information related to this field has been acquired from studies focusing on various animal species. However, our knowledge of the regulation of lipid transport in humans, especially at the intestinal level, remains elusive. Recently, we employed the model of jejunal organ culture to study the properties, development and regulation of nascent lipoprotein particles [5,6]. Our findings demonstrated that human jejunal explants in culture retain a few of the normal biochemical functions of adult intestine, such as the ability to elaborate complex lipid macromolecules associated with apoproteins. This efficient lipoprotein-lipid transport proved to be influenced by the developmental process and a certain number of hormones [5–7]. In particular, the addition of insulin to the culture medium significantly decreased the output of chylomicrons [8]. In this regard, the inhibition of triglyceride secretion by insulin has been demonstrated in perfused rat livers, rat hepatocytes and HepG2, a human hepatoma cell line [9–12].

The aim of this investigation was first to examine the effect of insulin on the de novo synthesis of intestinal apoproteins A-I and B, and second to determine whether changes in their production could be related to the level of apo A-I and apo B mRNA. Finally, the distribution of the two distinct forms of apo B (B-100 and B-48) and the molecular mechanism of their specific partitioning, referred to as apo B mRNA editing [13,14], were evaluated under the action of insulin.

2. Materials and methods

2.1. Intestinal specimens and culture conditions

Tissues from fetuses ranging from 17 to 20 weeks in age were obtained from normal elective pregnancy terminations. No tissue was collected from cases associated with known fetal abnormality or fetal death legal abortion. Studies were approved by the Institutional Human Subject Review Board. The entire small intestine was immersed in Leibovitz L-15 medium (room temperature) containing garamycin (40 μg/ml) and brought immediately to the culture room. The proximal half of the intestine excluding the first 3 cm was used and defined as jejunum.

The jejunum was cleansed of mesentery, split longitudinally, washed in culture medium, and cut into explants (3×7 mm). Five to seven explants were randomly transferred onto lens paper with the mucosal side facing up in each organ culture dish (Falcon Plastics, Los Angeles, CA). Six dishes were used for each experimental condition. An amount of medium (0.8 ml) sufficient to dampen the lens paper was added. Explants were cultured in serum-free Leibovitz L-15 medium according to the technique described previously [5–8]. After a 3 h stabilization period, the medium was changed with a fresh one containing a final amount of 0.13 μmol/ml of unlabeled oleic acid attached to albumin. The preparation of oleate/albumin complex was prepared as detailed previously [5–8]. Insulin was added at concentrations of 3 mU/ml and intestinal explants were cultured for 42 h.

2.2. De novo apolipoprotein synthesis: Pulse labeling of intestinal explants and immunoprecipitation procedure

Following the incubation period with unlabeled oleic acid to stimulate the synthesis of apoproteins, jejunal explants were washed twice with methionine-free Leibovitz medium. They were, then, incubated in the same medium, containing unlabeled oleic acid, for 45 min in the presence of [^{35}S]methionine (300 μCi/ml) with or without insulin. At the end of the labeling period, at 37°C, explants were washed (×3) and homogenized in phosphate-buffered saline (20 mM sodium phosphate, 145 mM NaCl, pH 7.4) containing 1% (w/v) Triton X-100, methionine (2 mM), phenylmethylsulfonyl fluoride (1 mM), and benzamidine (1 mM). Aliquots of tissue homogenates were precipitated with 20% trichloroacetic acid), and precipitates were washed three times with 5% trichloroacetic acid before the radioactivity was determined in a Beckman liquid scintillation spectrometer. The homoge-

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nates were also centrifuged (4°C) at 105 000 × *g* for 60 min in a 50-Ti rotor (Beckman, CA) and supernatants subsequently reacted with excess apoprotein polyclonal antibodies for 18 h at 4°C. The immunoprecipitation of apo A-I and apo B was carried out with polyclonal antibodies obtained commercially (Boehringer, Quebec, Canada). Pansorbin (Calbiochem, CA) was then added, and the mixture was reincubated at 20°C for 60 min. The immunoprecipitates were washed extensively and analyzed by a linear 4–20% acrylamide gradient preceded by a 3% stacking gel as described previously [7]. Gels were sectioned into 4-mm slices and counted after an overnight incubation at 20°C with 1 ml BTS-450 (Beckman) and 10 ml of liquid scintillation fluid (Ready Solv. NA, Beckman).

2.3. Sample extraction and RT-PCR analysis

After incubating explants in the presence or absence of insulin, total RNA was isolated as described by Chomczynski and Sacchi [15]. Single-strand cDNA was synthesized from the extracted and denatured RNA (5 min, 65°C) by the reverse transcriptase reaction, consisting of 1 mM dNTP (Pharmacia), 2.5 μM pd $(N)_6$ (Pharmacia), 200 U superscript II RNAse H transcriptase (Gibco), 8 U RNAse inhibitor (Boehringer-Mannheim, Montreal, Canada), 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl and 10 mM DTT. The mixture was incubated (10 min) at room temperature and reverse transcription was performed at 42°C for 2 h followed by inactivation (70°C for 15 min).

The PCR amplification was performed in a mixture containing 5 μl of reverse transcribed reaction, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 1 μM of each primer (lower and upper) and 2 U Tag DNA polymerase (Boehringer). The amplification of apo B and GAPDH was carried out in the same tissue with 30 cycles of denaturation at 94°C for 30 s annealing at 56.3°C for 90 s, and extension at 72°C for 60 s, with a final 10 min extension at 72°C. Apo A-I was amplified using 25 cycles and annealing at 62°C with the same conditions as above. PCR products (A-I, 196 bp; B, 238 bp; GAPDH, 384 bp) were separated with 6% polyacrylamide gel electrophoresis and quantified by Phosphorimager (Molecular Dynamics).

The PCR primers used in this study are:

Apo A-I:	minus:5′-CTG TGT ACG TGG ATG TGC-3′
	plus:5′-CCT TCT GTC TCC TTT TCC-3′
ApoB:	minus:5′-CCT TCT GTC TCC TTT TCC-3′
	plus:5′-CAA TCC CAT GTT CTG GAG-3′
GAPDH:	minus:5′-CCC ATC ACC ATC TTC CAG-3′
	plus:5′-CAT CAC GCC ACA GTT TCC-3′

2.4. Apo B mRNA editing assay

2.4.1. RNA preparation. Following organ culture technique, explants were washed extensively in ice-cold sterile saline, frozen in liquid nitrogen and stored at −80°C until processing for RNA extraction. RNA was extracted with buffer-saturated phenol/chloroform (1:1), and precipitated at −20°C. To remove contaminating residual nuclear DNA, total RNA was treated with RNase-free DNase (Promega) for 15 min at 37°C.

2.4.2. cDNA synthesis. 500–1000 ng total RNA in 10 μl was denatured for 5 min at 65°C and immediately chilled on ice. Following the addition of 10 μl of a mixture containing 0.5 mM dNTP (Pharmacia), 4 μM 3′-apo B oligo (5′ TTCAATGATATCAATAATA 3′), 12.5 U AMV reverse transcriptase (Boehringer Mannheim) and 8 U RNase inhibitor, a buffer solution (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KC) was added. The reverse transcription was performed at 42°C for 2 h.

2.4.3. PCR amplification. PCR amplification was carried out in 50 μl containing 5 μl reverse transcribed reaction, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP (Pharmacia), 1.6 μM apo B oligo (5′ GAGAAACTGACTGCTCTCAC 3′), 2 U Taq DNA polymerase, 30 cycles, each consisting of denaturation for 30 s at 94°C, annealing for 90 s at 54°C and extension for 1 min at 72°C. An extension at 72°C for 10 min was added to complete the amplification. PCR product (204 bp) was extracted from agarose gel electrophoresis, precipitated with ethanol and resuspended in 25 μl of sterile water.

2.4.4. Primer extension. Primer extension was performed according to a modification of the technique of Wu et al. [16]. Briefly, 5 μl of purified PCR product was denatured at 95°C for 5 min and kept on ice. To this sample was added a mixture (10 μl) containing 67 μM apo B-ext (5′ ATC ATA ACT ATC TTT AAT ATA CTG 3′), 0.33 mM of each dATP and dCTP (Pharmacia), 0.33 mM ddGTP, 33 μM dTTP, 15 μCi [α-^{32}P]TTP (NEN, Dupont), 12.5 U AMV reverse transcriptase (Boehringer Mannheim), 50 mM Tris pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT. The reaction was carried out at 42°C for 2 h. Two extension products (33 and 44 nucleotides for cDNA unedited and edited apo B mRNA, respectively) were fractionated on 15% PAGE (acrylamide/bisacrylamide, 19:1). The ratio of edited to unedited apo B mRNA was analyzed using a Phosphorimager (Molecular Dynamics). The validation of the primer extension and optimization of apo B mRNA editing were verified by controlling the critical number of cycles, using serial dilutions of total RNA from post-confluent cells and employing known standard amounts of CAA and TAA containing products.

2.5. Analyses

Lipid and lipoprotein synthesis were also assessed with [^{14}C]oleic acid as described previously [5,6]. The tissue homogenate was used for protein determinations according to Lowry et al. [17].

2.6. Statistical analysis

Results are reported as means ± S.E.M. The differences between means were assessed using the two-tailed Student's *t*-test.

3. Results and discussion

The mechanisms involved in the regulation of apolipoproteins have been studied extensively in the liver, since (1) this organ plays a central role in lipoprotein synthesis and secretion as well as in lipoprotein degradation [4], and (2) numerous hepatic models are available, including liver slices, perfused livers, hepatoma cells and primary cultures of hepatocytes [9–12,18]. Unlike the liver, the small intestine was evaluated to a lesser degree in similar functions, probably because of the limited number of models. Considering the well-known effects of insulin on protein and lipid synthesis, it was of interest to investigate its potential role in modulating lipoprotein and apolipoprotein biogenesis in human intestine. Thus, the aim of the current study was to determine the effect of insulin on the regulation of lipids, lipoproteins and apolipoproteins B-100, B-48 and A-I, using human jejunal explants cultured in serum-free medium.

The incorporation of [^{14}C]oleic acid into medium and cellular total lipids and main lipid classes was recorded over a period of 42 h. There was a linear output of neutral lipids and phospholipids over this period (data not shown). Total lipid content and triglycerides, in particular, were much higher in media than tissue, demonstrating active synthesis and secretion in cultures of jejunal explants (Fig. 1). Therefore, the intestinal organ culture provides a useful in vitro model for the investigation of lipoprotein processing in contrast to the limited secretion capacity of Caco-2, a human intestinal epithelial cell line, and constitutes a powerful tool to study fat transport mechanisms [19]. The most dramatic effect of insulin was the reduction of triglyceride and chylomicron secretion, confirming and extending our previous findings [8]. These observations are in agreement with the inhibitory effects of this hormone reported in cultured rat hepatocytes [9–12].

The next set of experiments was designed to examine the effect of insulin on the process of apolipoprotein biogenesis. Thus, the synthesis of apolipoproteins by jejunal explants was estimated by the incorporation of [^{35}S]methionine. Following the incubation, the apolipoproteins were immunoprecipitated, separated by SDS-polyacrylamide gel electrophoresis, identified on the basis of electrophoretic mobility (compared with coelectrophoresed native human plasma apolipoproteins as

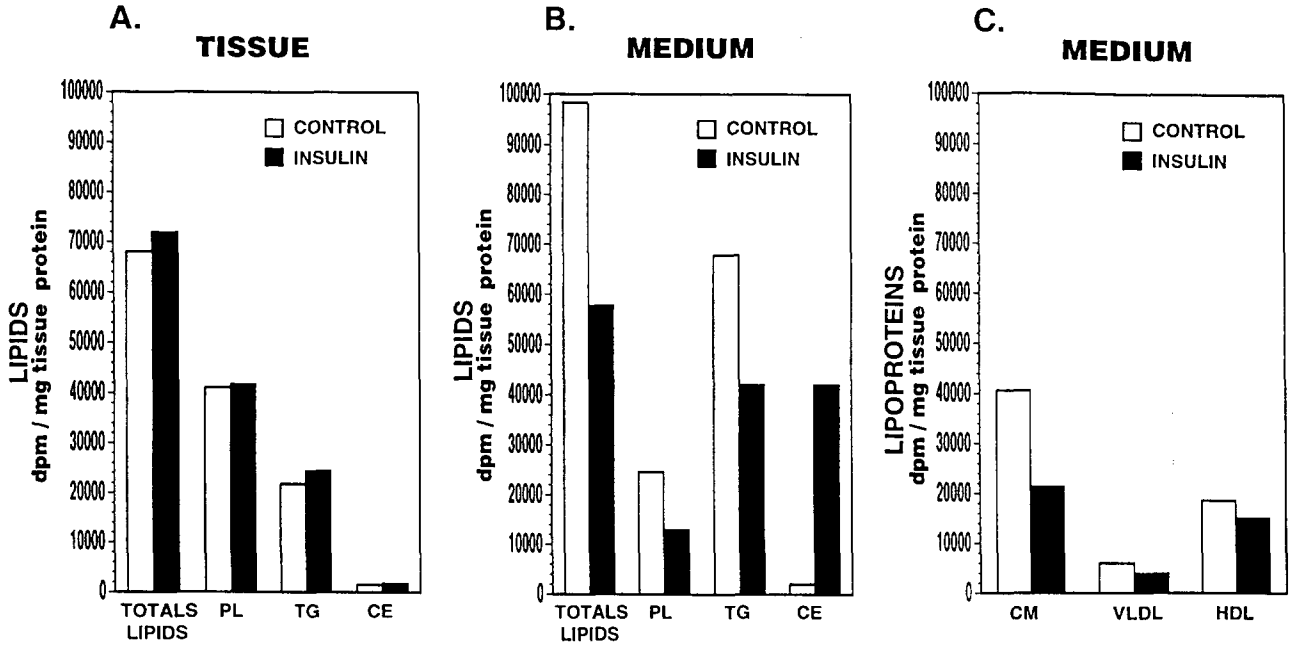

Fig. 1. Effect of insulin on lipid and lipoprotein secretion. After 42 h incubation with [^{14}C]oleic acid in serum-free Leibovitz L-15, in the absence and presence of insulin, the medium was recovered and chylomicrons, VLDL and HDL were separated by ultracentrifugation. Lipids were extracted, isolated by TLC and their radioactivity counted as described in Section 2. Results of tissue and medium lipids (A,B) as well as lipoproteins (C) are expressed as dpm/mg protein in this representative experiment. TG, triacylglycerol; PL, phospholipid; CE, cholesteryl ester.

well as stained molecular weight standards) and then counted. Fig. 2 shows a representative gel scan, which revealed that the jejunal organ culture produced apo A-I as the predominant apolipoprotein. In all experiments, apo B-48, of lower molecular weight, was the major apo B form. Incubation of jejunal explants in the presence of insulin results in a trend of increased [^{35}S]methionine incorporation into newly synthesized apo B (Fig. 2B). However, the addition of insulin produced a significant decrease in the secretion of apo B-48 and apo B-100 (Fig. 2C). On the other hand, insulin had little effect on

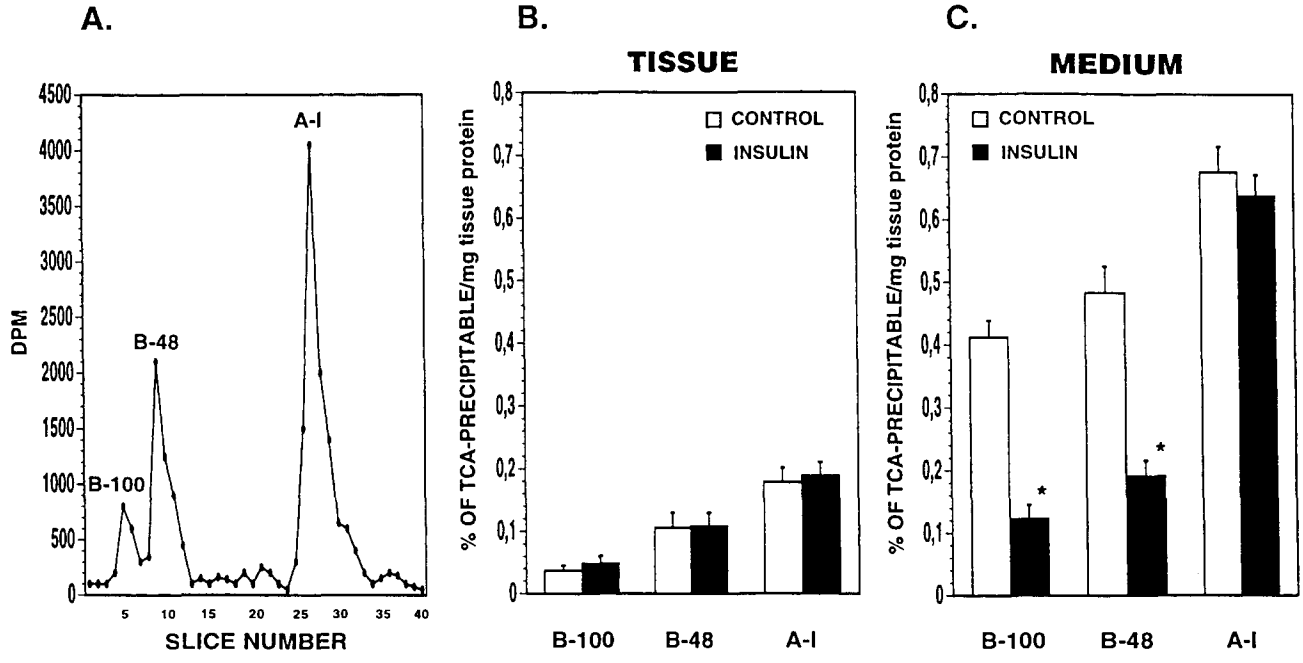

Fig. 2. Profile of ^{35}S-labeled apolipoproteins A-I and B synthesized by cultured jejunal explants. Following 42 h incubation with unlabeled oleic acid in the absence and presence of insulin, jejunal explants were incubated for 45 min with methionine-free medium containing unlabeled oleic acid and [^{35}S]methionine with or without insulin. Apolipoproteins were immunoprecipitated and analyzed by SDS-PAGE. Data from a representative experiment are illustrated in A. Means ± S.E.M. (of 10 experiments carried out in triplicates) for tissue and media (expressed as a percent to total [^{35}S]methionine labeled protein/mg tissue protein) are presented in B and C, respectively. *Results are significantly different from the no insulin condition at $p < 0.01$.

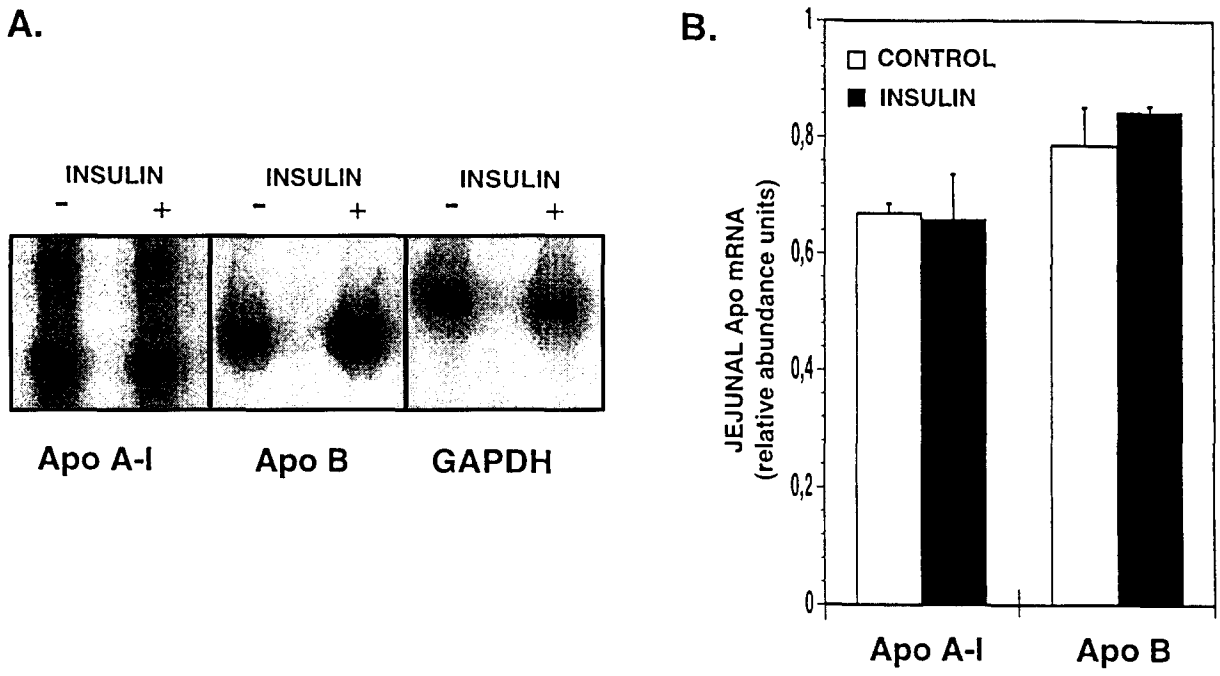

Fig. 3. Effect of insulin on apoprotein transcript levels in jejunum explants from 16–19-week-old fetuses. Explants were incubated and mRNA determined as indicated in Section 2. (A) Representative effect of insulin on the expression of apolipoproteins. (B) Relative levels (means ± S.E.M.) of apolipoprotein mRNA measured in the jejunal explants of 7 individual fetuses (carried out in triplicates) by the technique described in Section 2. Data are expressed as average ratio values of control apo mRNA/control GAPDH mRNA and insulin apo mRNA/insulin GAPDH mRNA, GAPDH being the housekeeping gene.

apo A-I synthesis in parallel with the unchanged production of HDL. These results suggest that the observed reduction in intestinal triglyceride and chylomicron output in vitro, subsequent to insulin administration, may reflect a direct action of insulin on apo B secretion. In similar fashion, the addition of insulin to primary cultures of rat hepatocytes resulted in the failure of a substantial portion of newly synthesized apo B to be secreted [20].

In order to determine whether the reduction in apo B synthesis caused by insulin was related to decreases in intestinal apo B mRNA, RT-PCR analysis was performed. Interestingly, the representative autoradiogram of these gels illustrated in Fig. 3, as well as the mean of 7 distinct experiments, indicates that insulin had no significant effect on the abundance of apo B mRNA. Under our experimental conditions, the relative apo B mRNA levels in the presence of insulin were close to control values. This suggests that in cultured jejunal explants, the expression of the apo B gene is constitutive and that co- or post-translational mechanisms may be responsible for the regulation of apo B formation. The translational efficiency and stability of apo B mRNA may be involved. Intracellular degradation of apo B has been proposed as a regulatory event for VLDL secretion by the liver. Studies in rat hepatocytes indicated significant degradation of apo B mediated by insulin [21,22]. In agreement, little degradation was noted when insulin was absent under similar conditions [20]. However, additional investigation is still necessary to assess whether increased apo B degradation, in the presence of insulin, occurs in human intestinal explants. Some workers also stress the need to focus on the translational efficiency of apo B mRNA transcripts. For example, Adeli and Theriault [23] have observed a reduction in apo B synthesis in insulin-treated HepG2 lysates, due to a lower translational efficiency of apo B mRNA.

Black and Ellinas [24] have also investigated the effects of insulin on apo B synthesis in jejunal explants from newborn piglets. No influence of insulin was recorded in these experiments, suggesting that the modulation of apo B synthesis by insulin may depend on both the stage of development and the species studied. Consequently, our results may not be directly applicable to mature intestinal function.

Apolipoprotein B is required for the intracellular assembly and secretion of triglyceride-rich lipoproteins [20,25]. There are two translation products of the apo B gene: human apo B-100 is synthesized predominantly by the liver and human apo B-48 is produced by the intestine through the newly mRNA editing mechanism [13,14]. In our study, the profile of apo B mRNA editing was determined by the triplicate analysis of separate jejunal explants deriving from 7 fetuses. A representative autoradiograph of primer extension products isolated from editing reactions is shown in Fig. 4. By 17–20 weeks of gestation, editing reached a level of 50% UAA. Similar proportions in the ratio of newly synthesized apo B-48 and apo B-100 were obtained. In contrast, apo B mRNA editing was not detectable in triplicate analyses of RNA from HepG2 cells used as a negative control. In the human intestinal experimental model, we found that the proportion of edited apo B mRNA in insulin-treated culture was unchanged compared with that of controls. Therefore, insulin, unlike other metabolic and hormonal factors in rats, apparently had no detectable effect on apo B mRNA editing in humans. Thyroid hormone treatment in hypothyroid rats [26] and carbohydrate refeeding diet following fasting [27] modify the amount of hepatic apo B-48 mRNA. Apo B

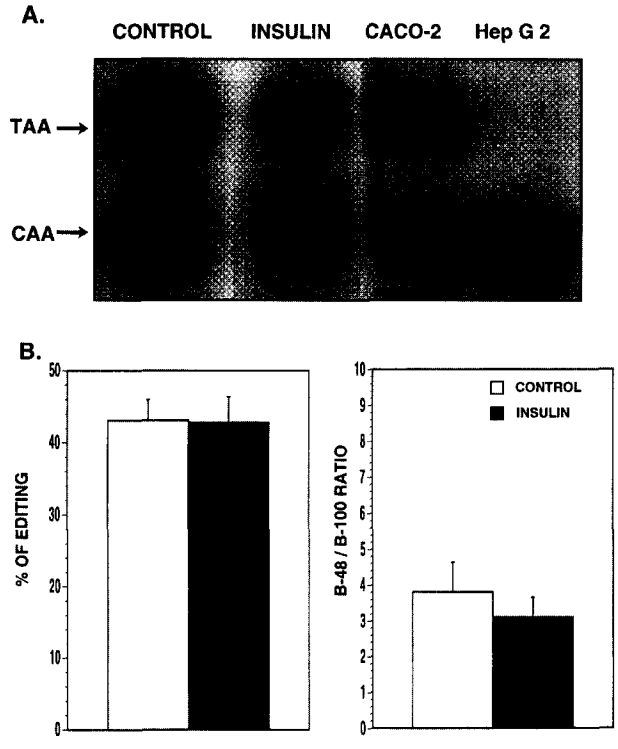

Fig. 4. Apolipoprotein B mRNA editing detected by RNA primer extension analysis in human intestinal explants. The migration of non-edited (CAA) and edited (TAA) is shown. Caco-2 and Hep G2 cells are used as positive and negative controls, respectively. (A) Representative result from 7 separate experiments. The ratio of B-48/B-100 and edited to unedited apo mRNA was determined using a Phosphorimager and is presented in B.

mRNA editing is a developmentally regulated process involving apo B expression. In the postnatal rat, swine and human, the adult small intestine produces predominantly apo B-48 because of the activity of this mRNA editing, while editing activity is reduced earlier in gestation and increases dramatically to near adult levels, just before parturition [28,29].

In our study, the synthesis of apo A-I was not significantly altered in the presence of insulin. In contrast, Masumoto et al. [30] reported that higher concentrations of insulin inhibit apo A-I production in cultured rat hepatocytes, whereas Elshourbagy et al. [31] observed a 2-fold increase in apo A-I mRNA in rat hepatocytes. These contradictory results, however, could be due to the use of different models (intestine vs liver) and species (humans vs rats).

It has been suggested that cholesteryl ester (CE), the other core lipid of apo B-containing lipoproteins, is a critical regulator of apo B secretion [32]. In cultured jejunal explants, cholesteryl ester synthesis was limited and unaffected by insulin. It is likely, therefore, that CE is not an important determinant in TG-rich lipoprotein secretion, confirming the data of Ginsberg's laboratory [33].

In summary, insulin caused a significant decrease in chylomicron production, concomitant with a reduction in the secretion of de novo apo B-100 and apo B-48 synthesis. There were no major changes in HDL lipids or apo A-I. The inhibition of apo B output by insulin treatment was not due to altered mRNA level. This may implicate possible co- or post-translational modification of apo B, resulting in impaired chylomicron formation. The responsiveness of jejunal explants to the addition of insulin provides further evidence for the utility of intestinal organ culture in studying the regulation of human lipoprotein processing.

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