

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION



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Arterioscler. Thromb. Vasc. Biol. 1999;19:1051-1059

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 75214

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ISSN: 1524-4636

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Niacin Accelerates Intracellular ApoB Degradation by Inhibiting Triacylglycerol Synthesis in Human Hepatoblastoma (HepG2) Cells

Fu-You Jin, Vaijinath S. Kamanna, Moti L. Kashyap

Abstract—The mechanism by which the potent drug niacin decreases apoB-containing atherogenic lipoproteins and prevents coronary disease is unclear. Utilizing human hepatoblastoma (HepG2) cells as an in vitro model, we have examined the effect of niacin on intracellular degradation of apoB and the regulatory mechanisms involved in apoB processing. Niacin significantly increased apoB degradation in a dose- and time-dependent manner. Treatment of HepG2 cells with calpain inhibitor I [N-acetyl-leucyl-leucyl-norleucinal (ALLN), an inhibitor of certain protease-mediated apoB degradation], did not alter niacin-induced apoB degradation. Niacin decreased inhibition of oleate-mediated apoB degradation. Niacin dose-dependently inhibited the synthesis of both fatty acids and triacylglycerol (TG) by 20% to 40% as determined by the incorporation of ^{14}C -acetate and ^3H -glycerol into fatty acids and TG, respectively. Incubation of HepG2 cells with niacin significantly inhibited (by 12% to 15%) fatty acid esterification to produce TG as assessed by the incorporation of ^3H -oleic acid into TG. ^{14}C -acetate incorporation into cholesterol and phospholipids was unchanged. The activity of microsomal triglyceride transfer protein (MTP), a carrier protein for lipids, was not altered by pretreatment of cells with niacin. ApoB mRNA expression and ^{125}I -LDL protein uptake were also unchanged. These data indicate that niacin accelerates hepatic intracellular post-translational degradation of apoB by selectively reducing triglyceride synthesis (through inhibiting both fatty acid synthesis and fatty acid esterification to produce TG) without affecting ALLN-inhibitable protease- or MTP-mediated intracellular apoB processing, resulting in decreased apoB secretion and hence lower circulating levels of the atherogenic lipoproteins. (*Arterioscler Thromb Vasc Biol.* 1999;19:1051-1059.)

Key Words: atherosclerosis ■ coronary disease ■ hepatic apoB degradation ■ niacin ■ fatty acid ■ triacylglycerol

ApoB is the major protein of VLDL, IDL, LDL, and Lp(a). Elevated levels of these apoB-containing lipoproteins, especially LDL, are associated with accelerated atherosclerosis, and its reduction results in a reduction in these complications. A growing body of evidence indicates that a large amount of de novo-synthesized apoB is not secreted but rather degraded intracellularly by cultured hepatocytes.¹⁻⁵ Furthermore, recent studies have shown that translocation of apoB across the endoplasmic reticulum (ER) regulates the degradative pathway and the subsequent secretion in the plasma.⁶

In addition to the intracellular processes involved in translocation and degradative mechanisms, the cotranslational association of core lipids with apoB to form nascent lipoproteins appears to play an important role in regulating hepatic secretion of apoB-containing lipoproteins.⁷ For example, preincubation of HepG2 cells with oleate was shown to markedly reduce apoB degradation, suggesting that the

newly synthesized lipids may facilitate the translocation of apoB into the ER lumen, and thus reduce the availability of apoB for degradation by proteases localized either in the cytosol or on the cytosolic side of the ER membrane.^{8,9} Furthermore, microsomal triglyceride transfer protein (MTP), a lipid carrier protein within the membrane, has been suggested to play an important role in facilitating the secretion of apoB-containing lipoproteins. Mutations in the gene encoding for the 97-kDa subunit of MTP have been implicated in a complete lack of the secretion of apoB-containing lipoproteins in rare autosomal recessive abetalipoproteinemia.^{10,11} Using transfection of COS-1 cells with truncated forms of apoB and MTP, recent studies indicated that the expression of MTP in heterologous cell systems may be sufficient to direct the assembly and secretion of apoB-containing lipoproteins.¹²

Although these complex cellular mechanisms regulating apoB secretion are fairly well known, the intracellular processes by which lipid-lowering drugs exert their action to

Received February 23, 1998; revision accepted October 6, 1998.

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Part of this work was presented in abstract form at the Annual Meeting of the American Heart Association, in New Orleans in November 1996, and done during a visiting professorship at the National University of Singapore by Dr Kashyap.

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decrease atherogenic plasma apoB-containing lipoproteins are not clearly understood. For example, despite the wide usage of nicotinic acid (niacin) to decrease plasma LDL levels and to treat atherosclerotic cardiovascular disease,¹³⁻¹⁷ the mechanism of action of niacin to lower LDL levels has not been fully defined. Earlier studies suggested that niacin, by decreasing the production of VLDL, may lower plasma levels of VLDL and LDL.^{13,18,19} Additional reports indicated that the ability of niacin to decrease the transport (or production) of VLDL-triglycerides may be a predominant mechanism to lower triglyceride levels.¹³ Nevertheless, these earlier studies did not address the role of niacin in modulating the synthesis and post-translational events involved in hepatic secretion of apoB-containing lipoproteins.

Thus, using a human hepatoblastoma cell line (HepG2) as an *in vitro* model, we examined the effects of niacin on intracellular apoB degradation and regulatory factors (such as protease-mediated degradation, microsomal triglyceride transfer protein and synthesis of fatty acids and lipids) involved in hepatic apoB secretion. This *in vitro* model has been shown to be useful in studies related to the hepatic secretion of apoB-containing lipoproteins.³ The data presented indicate that niacin increased intracellular apoB degradation by selectively decreasing hepatic synthesis of triglycerides without affecting certain protease- or MTP-mediated intracellular apoB processing.

Methods

Materials

Tissue culture materials, media, BSA (essentially fatty acid-free), oleic acid (sodium salt), ALLN, and niacin were obtained from Sigma Chemical Company. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories. L-[4,5-³H]leucine and ³²P-nucleotide were purchased from Amersham Corporation. Human hepatoblastoma cell line HepG2 and human apoB DNA probe were obtained from American Type Culture Collection (Rockville, MD). Polyclonal antibody for human apoB was obtained from Boehringer Mannheim Biochemicals. Protein A-Sepharose CL-4B was purchased from Pharmacia LKB Biotechnology Inc. SDS-PAGE reagents were obtained from BIO-RAD Laboratories. Niacin stock solution was prepared in the culture medium (DMEM). The pH was adjusted to 7.4 with 1N NaOH, and appropriate concentrations of niacin were added to the culture dishes. All other chemicals used were of analytical grade.

HepG2 Cell Culture and Pulse-Chase Studies for ApoB Degradation

HepG2 cells were grown in Costar T-75 tissue culture flasks with 15 mL of high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. HepG2 cells were preincubated for 48 hours with varying concentrations of niacin (0 to 3.0 mmol/L) in DMEM containing 10% FBS media. Cells were washed twice with PBS and preincubated at 37°C in leucine-free medium containing 1.5% BSA (essentially fatty acid-free) and the same concentrations of niacin for 2 hours, then pulsed with L-[4,5-³H]leucine (100 µCi/mL) for 15 minutes at 37°C. After the pulsing period, cells were washed with PBS and chased for 0 to 180 minutes as described in individual figure legends. At each time point, media were collected and cells washed with PBS and digested in an ice-cold lysis buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.06 mol/L sucrose, and protease inhibitors, pH 7.4). Aliquots of cell lysate and chase medium were used for apoB immunoprecipitation or SDS-PAGE analysis. During the course of the studies we monitored the potential toxic effects of niacin on HepG2 cells by measuring cell viability by

trypan blue exclusion and DNA or protein content. Niacin, at various doses (up to 3 mmol/L) had no effect on cell viability. During the incubation period of 48 hours, ≈1% to 2% of the cells were stained with trypan blue in both control and 0.25 to 3 mmol/L niacin-treated cells. Additionally, no significant differences in DNA or protein content were observed between control and niacin-treated cells.

Immunoprecipitation of ApoB and SDS-PAGE Analysis

Aliquots (500 µL) of medium or cell lysate were mixed with 500 µL of NET buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L Tris, pH 7.4, 0.5% Triton X-100, and 0.1% SDS) containing excess monospecific sheep anti-human apoB antiserum and incubated overnight at 4°C on a rocking platform shaker. Protein A-Sepharose CL-4B beads (100 µL of a 5% solution in NET buffer) was added to each tube and the incubation was continued for an additional 3 hours. The antigen/antibody complex bound to protein A-Sepharose CL-4B beads was collected by brief centrifugation at 12 000g, washed 5 times with 1 mL of NET buffer and reconstituted in 100 µL of electrophoresis buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). ApoB was removed from the protein A-Sepharose beads by boiling for 4 minutes and the released apoB in the supernatant was collected by centrifugation and quantified by liquid scintillation counting. An aliquot of apoB released was subjected to electrophoresis through a 5% to 15% gradient polyacrylamide gel containing SDS. The gel was incubated in fluorographic solution (Amplify) for 1 hour, dried, and exposed to x-ray film to detect apoB protein bands. Cell protein was determined by Lowry's method using BSA as a standard.

Intracellular ApoB Degradation in the Presence of Oleate or ALLN and Niacin

HepG2 cells were grown in 6 well plates (35 mm) for 3 days to attain 70% to 80% confluence in DMEM+10% FBS media. Cells were preincubated in the absence or presence of varying concentrations (0 to 3.0 mmol/L) of niacin for 48 hours. After the incubation, cells were washed twice with PBS and incubated in 1 mL of serum-free medium containing the same concentrations of niacin, 1.5% fetal bovine albumin (FBA) and either 0.4 mmol/L oleate for 2 hours or 40 µg/mL calpain inhibitor I (ALLN) for 1 hour. Cells were washed twice with PBS and the medium was replaced with leucine-free DMEM containing 100 µCi of ³H-leucine. After 15 minutes of pulse labeling, the medium was removed, then cells were washed twice with PBS and chased for 1 hour in serum-free DMEM. During the pulse-chasing period, the same concentrations of niacin and oleate or ALLN were present in the media. ApoB degradation was assessed as described above using cell lysate and chase medium.

Uptake of LDL by HepG2 Cells Treated With Niacin

Studies examining the uptake of LDL by HepG2 cells were performed by utilizing radiolabeled LDL. Radioiodination of LDL total protein was carried out by incubating freshly isolated LDL ($d=1.03$ to 1.05 g/mL) with carrier-free ¹²⁵I as described earlier by McFarlane.²⁰ After the iodination, unreacted ¹²⁵I was removed by gel filtration followed by exhaustive dialysis against PBS. The specific activity of ¹²⁵I-LDL protein was 186 cpm/ng protein. Uptake studies were initiated by preincubating HepG2 cells with varying concentrations of niacin (0 to 3.0 mmol/L) for 48 hours at 37°C. The media were replaced with fresh DMEM containing fetal bovine albumin (5 mg/mL) and ¹²⁵I-LDL (50 µg protein) was added. After 6 hours of incubation at 37°C, cell monolayers were washed thoroughly (4 to 5 times with PBS) and digested with 1N sodium hydroxide solution. An aliquot was used for radioactivity measurement. The uptake of radiolabeled LDL particles by HepG2 cells was expressed in terms of cellular protein.

Northern Blot Analysis of ApoB mRNA

HepG2 cells were preincubated with varying concentrations of niacin (0 to 3.0 mmol/L) for 48 hours. Total RNA was isolated from HepG2 cells using the protocol of Chomczynski and Sacchi.²¹ Twenty micrograms of total RNA was loaded into individual wells of a 1.2%

Effect of Niacin on ApoB Intracellular Degradation and Secretion by HepG2 Cells

Niacin Conc. (mmol/L)	ApoB Retained (CPM/mg cell protein)				Total ³ H-ApoB (Cell+Medium)	% Decay
	Intracellular	<i>P</i> Value	Secretion	<i>P</i> Value		
Control	7455±240		3981±122		11 436±362	0
0.25	7480±179	0.8921	3598±25	0.0061	11 078±204	3.1
0.50	5105±148	<0.0001	3264±241	0.0102	8369±389	26.8
1.00	5005±138	<0.0001	3046±121	0.0007	8051±259	29.6
2.00	4448±410	0.0004	3144±118	0.0010	7592±528	33.6
3.00	4323±235	<0.0001	3042±49	0.0003	7365±284	35.6

Cells were preincubated with varying concentrations of niacin (0 to 3.0 mmol/L) for 48 hours at 37°C. The cells were washed twice with PBS and incubated with leucine-free medium containing 1.5% BSA and niacin for 2 hours, pulse-labeled with ³H-leucine for 15 minutes, and chased for 60 minutes. At the end of chase period, medium was collected and cells were lysed and the amount of radiolabeled-apoB in both of medium and cell lysate was measured by immunoprecipitation.

Data are mean±SE of 3 separate experiments done in triplicate. Statistical significance was compared with results of control.

agarose gel containing formaldehyde and electrophoresed,²² the RNA transferred onto MSI nylon membranes, UV-cross-linked and hybridized overnight with the [α -³²P]dCTP-labeled cDNA probe for human apoB. After washing the membrane, mRNA detection was performed by autoradiography. Blots were then rehybridized with human GAPDH cDNA probe as an internal control to assess RNA quantity and integrity. mRNA signals were quantified by densitometric scanning of autoradiographic bands (LKB laser densitometer, Pharmacia, LKB Biotech) and normalized with GAPDH signals.

Microsomal Triglyceride Transfer Protein (MTP) Activity Assay

MTP activity was measured by using an MTP Assay Kit according to manufacturer's instructions (Diagenescent Technologies, Inc). Briefly, HepG2 cells were treated with niacin at varying concentrations (0 to 3.0 mmol/L) for 48 hours. After incubation, cells were washed with PBS and collected in homogenization buffer (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl flouride and 20 μ g/mL leupeptin). The cell suspension was sonicated and 50 μ g of homogenate protein was used in the assay. MTP assay was performed by incubating 10 μ L of donor and 10 μ L of acceptor solutions with 5 μ L HepG2 cell homogenate (50 μ g protein, MTP source) in a total volume of 0.5 mL buffer (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA) and incubated for 6 to 12 hours at 37°C. The MTP activity was calculated by measuring the fluorescence at excitation wavelength of 465 nm and emission wavelength of 535 nm.

Fatty Acid, Cholesterol, Cholesteryl Ester, Phospholipid and Triacylglycerol (TG) Synthesis Assay

HepG2 cells were preincubated for 48 hours with varying concentrations of niacin (0 to 3.0 mmol/L). Cells were washed twice with serum-free DMEM and incubated at 37°C in the serum-free DMEM medium containing 5 μ Ci/mL ³H-glycerol (for TG synthesis) or 1 μ Ci/mL ¹⁴C-acetate (for fatty acid, TG, cholesterol, cholesteryl ester or phospholipid synthesis) or 1 μ Ci/mL of ³H-oleic acid (for assessing fatty acid esterification to produce TG) and equal concentrations of niacin for 4 hours. After incubation, the medium was removed and cells were washed thoroughly with PBS. Total cellular lipids were extracted by chloroform/methanol (2:1, vol/vol), and the lipid extract was dried under nitrogen and reconstituted in 100 μ L hexane. Radiolabeled lipids were separated by thin-layer chromatography (TLC) on silica-coated plates using hexane/diethylether/acetic acid (80:20:1, vol/vol) as a developing solvent. Lipids were visualized by exposure to iodine vapors, the bands corresponding to authentic lipid standards (eg, fatty acids, TG, cholesterol, cholesteryl ester, or phospholipids) were scraped and the radioactivity was measured by liquid scintillation counting and expressed as CPM/mg cell protein.

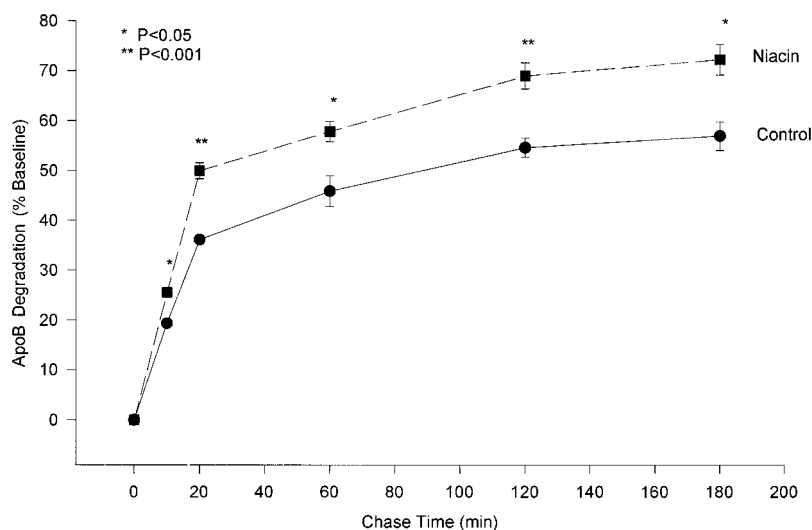
Statistical Analysis

The mean data presented are average values for each set of experiments done in triplicate. Each experiment was repeated 3 times. Statistical significance was calculated by using the Student's *t* test and a *P* value <0.05 was considered significant.

Results

ApoB degradation studies performed in HepG2 cells (pulsed with ³H-leucine for 15 minutes, chased for 1 hour) indicated that the preincubation of cells with niacin (0 to 3 mmol/L) for 48 hours dose-dependently decreased apoB accumulation in the media by 9% to 23% during a 1-hour chase period (Table). To understand intracellular mechanisms of hepatic apoB secretion in response to niacin treatment, we have carried out a series of studies using HepG2 cells as an in vitro model that examined various intracellular regulatory events involved in apoB degradation and secretion. Pulse-chase experiments (pulsed for 15 minutes) performed for 1 hour indicated that the preincubation of HepG2 cells with varying concentrations of niacin (0 to 3 mmol/L) for 48 hours dose-dependently increased apoB intracellular degradation and decreased subsequent secretion of apoB into the culture media (Table) compared with controls. Niacin, as low as 0.5 mmol/L, significantly increased total (intracellular plus media) apoB degradation by 26.8% when compared with controls and the maximum degradation of 35.6% was noted at 3 mmol/L of niacin (Table). Time-course studies examining the effect of niacin on apoB degradation at varying incubation times (4 to 48 hours) indicated that niacin, up to 8 hours incubation, had no significant effect, but niacin, at 24 hours incubation, resulted in a marginally significant effect on apoB degradation (data not shown). The maximal effect on apoB degradation was observed with 48 hours of incubation with niacin, hence a 48-hour incubation period with niacin was used for all the experiments.

Incorporation of ³H-leucine into intracellular apoB at 0 minutes chase was measured as an index of apoB synthesis. During the 15-minute pulse period, niacin produced no significant change in the de novo synthesis of apoB. The apoB synthesis (³H-leucine incorporation, cpm/mg cell protein) in control and niacin-treated cells are 20 593±1054 and 18 510±1067, respectively (data shown in Figure 1 legend). The apoB synthesis (cpm) in niacin-treated cells were



respectively. Data are mean \pm SE of 3 separate experiments done in triplicate. Statistical significance was compared with results of controls.

\approx 15% lower than control. Although this was not significant statistically, the lower value could represent some degradation of apoB during the 15-minute leucine pulse. Additional pulse-chase experiments (pulse, 15 minutes; chase, 0 to 180 minutes) were performed to determine the effect of niacin on total apoB degradation (intracellular plus secreted into media) at various chase periods (0 to 180 minutes). The results from these studies indicated that the pretreatment of HepG2 cells with niacin (1.5 mmol/L) for 48 hours significantly increased the total degradation of apoB during chase periods of 20 to 180 minutes when compared with the respective controls (Figure 1). Intracellular apoB degradation, as measured by percent of total initial cellular apoB decayed (intracellular plus secreted into the media), at as early as 20 minutes chase period, was markedly higher in niacin-treated cells as compared with controls (apoB degradation: control = $36.2 \pm 0.58\%$, niacin = $50.0 \pm 1.15\%$, $P = 0.004$); apoB degradation at 3 hours chase period in control and niacin-treated cells was $56.8 \pm 1.85\%$ and $72.2 \pm 1.84\%$, respectively (Figure 1, $P = 0.004$). Further confirmatory tests for apoB degradation using immunoprecipitation techniques were performed by separating apoB bands via SDS-PAGE and semi-quantifying the degradation by fluorography of apoB bands. Results indicated that, at each chase period (10 to 180 minutes), niacin-treated cells had markedly reduced full-length apoB bands when compared with the respective chase-time point of control cells, indicating increased intracellular apoB degradation by niacin (Figure 2). However, no degraded apoB bands (ie, smaller molecular weight bands) were seen on the fluorograms of either control or niacin-treated cells. The percent of apoB remaining at various chase periods (10 to 180 minutes) in control and niacin-treated cells was measured by densitometric scanning of fluorograms. The results indicated that the percentage values of apoB remaining in control cells were: 80.7%, 40.1%, 21.2%, 10.7%, and 7.2% during chase periods of 10, 20, 60, 120, and 180 minutes, respectively; whereas the percentage values of apoB remaining in niacin-treated cells were: 56.0%, 33.4%, 16.2%, 7.5%, and 4.8% during chase periods of 10, 20, 60, 120, and 180 minutes, respectively.

Figure 1. Effect of niacin on apoB degradation by HepG2 cells. Cells were preincubated with or without niacin (1.5 mmol/L) for 48 hours at 37°C. Cells were washed twice with PBS and incubated with leucine-free medium containing 1.5% BSA and niacin (1.5 mmol/L) for 2 hours, pulse-labeled with ^3H -leucine for 15 minutes. After the incubation, cells were washed with PBS and chased for 0 to 180 minutes with normal DMEM. At the end of each chase period, media and cells were collected, and radiolabeled-apoB retained in the medium and cell lysate was measured by immunoprecipitation. Total apoB degradation (cellular and medium) was expressed as percent reduction in 0 minutes chase apoB radioactivity. Incorporation of ^3H -leucine into intracellular apoB at 0 minutes chase served as a measure of apoB synthesis. The apoB syntheses (^3H -leucine incorporation, cpm/mg cell protein) in control and niacin-treated cells were $20\,593 \pm 1054$ and $18\,510 \pm 1067$ (not statistically significant),

Because the results presented in the Table and Figure 1 show increased apoB degradation in niacin-treated cells, additional studies were designed to delineate regulatory intracellular mechanisms associated with apoB degradation. Initially, pulse-chase apoB degradation experiments were done in control and niacin-treated cells (48 hours) in the presence of ALLN, a protease inhibitor, to determine whether niacin-induced apoB degradation is mediated through ALLN-inhibitable protease-dependent pathways. In these experiments, HepG2 cells were preincubated with niacin for 48 hours, treated with ALLN for 1 hour and pulse-chase experiments were performed for 0 to 60 minutes chase period. Congruent with earlier studies,²³ treatment of HepG2 cells with ALLN significantly decreased apoB degradation when compared with control cells without ALLN treatment, suggesting the feasibility of our experimental conditions used to measure apoB degradation. The apoB degradation (including cell and medium), as measured by percentage decay of initial during 10 to 60 minutes chase period, was 15.1% to 46.8% and 1.0% to 25.6% in control and ALLN-treated cells, respectively. To confirm the role of ALLN-mediated inhibition of apoB degradation in niacin-treated cells, intracellular

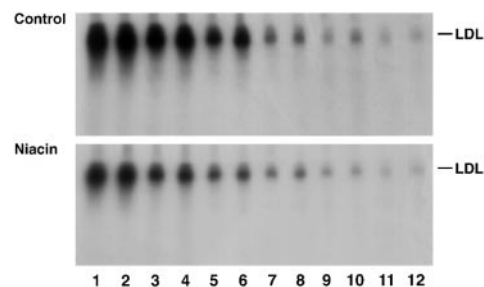


Figure 2. Effect of niacin on intracellular apoB content by SDS-PAGE analysis: Cells were incubated with or without niacin (1.5 mmol/L) for 48 hours, pulsed with $100\ \mu\text{Ci}/\text{mL}$ of ^3H -leucine for 15 minutes, and chased for 0 to 180 minutes. Cellular ^3H -apoB was immunoprecipitated and analyzed by SDS-PAGE (5% to 15% gradient gel). Lanes 1 to 12 represent apoB signal from cell extracts of 0, 10, 20, 60, 120, and 180 minutes chase period (in duplicates), respectively. The marker (—LDL) shows the location of Coomassie Blue-stained standard apoB of

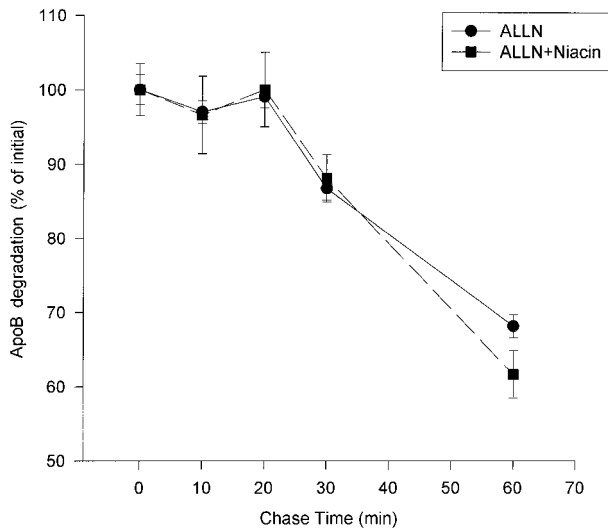


Figure 3. Effect of ALLN on apoB degradation in control and niacin-treated HepG2 cells. Cells were preincubated with or without niacin (1.5 mmol/L) for 48 hours at 37°C. The cells were washed twice with PBS and incubated with leucine-free medium containing 1.5% BSA and ALLN (40 µg/mL) and an equal concentration of niacin for 1 hour, pulse-labeled with ³H-leucine for 15 minutes, and chased for 0 to 60 minutes. At the end of the chase period, media and cells were collected, and the total radiolabeled apo-B (cell and media) retained was measured by immunoprecipitation and expressed in terms of total cell protein. ApoB degradation was expressed as the percent reduction in initial apoB-radioactivity (0 minutes chase). Data are mean±SE of 3 separate experiments done in triplicate.

apoB degradation in HepG2 cells treated with ALLN alone or in the presence of ALLN and niacin was compared at various chase periods. As shown in Figure 3, the apoB degradation (as measured by percentage decay of initial apoB in cell plus medium) in ALLN and ALLN plus niacin-treated cells was not significantly different. These results suggest that ALLN-induced effects on apoB degradation were not altered by niacin treatment of cells.

Because oleate has been shown to inhibit early apoB degradation, further experiments were designed to examine whether niacin could alter oleate-mediated effects on apoB degradation to determine the involvement of lipids in niacin-induced intracellular apoB processing. The incubation of niacin-pretreated cells with oleate significantly increased cellular apoB degradation when compared with oleate treatment alone (Figure 4). The increased intracellular apoB degradation induced by varying concentrations of niacin (0.5 to 3 mmol/L) was significantly inhibited by coincubation with oleate (percentage apoB degradation at niacin doses of 0.5 to 3 mmol/L: niacin alone=26.8% to 35.6%, niacin plus oleate=9.8% to 13.4%). These data indicate that niacin-induced apoB degradation may be dependent on the pathways involving the synthesis and association of lipids before apoB processing.

To determine the direct effects of niacin on cellular lipid synthesis, experiments were carried out by examining the incorporation of ³H-glycerol and ¹⁴C-acetic acid into triglycerides, cholesterol and cholesteryl esters. As shown in Figure 5A and 5B, the incubation of HepG2 cells with varying concentrations of niacin (0 to 3 mmol/L) dose-dependently inhibited fatty acid and TG synthesis by 20% to 40% as determined by the incorporation of ³H-glycerol and ¹⁴C-acetic acid into newly synthesized lipids (data not shown).

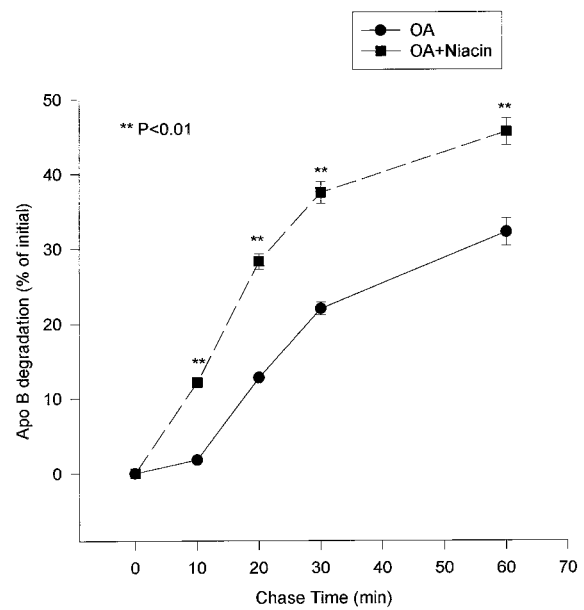


Figure 4. Effect of oleic acid on niacin-induced apoB degradation by HepG2 cells. Cells were preincubated with or without niacin (1.5 mmol/L) for 48 hours at 37°C. The cells were washed twice with PBS and incubated with leucine-free medium containing 1.5% BSA, 0.4 mmol/L oleic acid, and niacin for 2 hours, pulse-labeled with ³H-leucine for 15 minutes, and chased for 0 to 60 minutes. At the end of each chase period, cells were lysed and radiolabeled apoB retained was measured by immunoprecipitation and expressed in terms of total cell protein. ApoB degradation was expressed as the percent reduction in initial apoB-radioactivity (0 minutes chase) decayed from the cell. Data are mean±SE of 3 separate experiments done in triplicate. Statistical significance was compared with results of controls.

synthesized fatty acids or TG. Similarly, the incubation of HepG2 cells with niacin significantly (~20% to 40%) inhibited the incorporation of ³H-glycerol into TG (Figure 6A). Parallel to the results of apoB degradation in the presence of niacin plus oleate (Figure 3), niacin, in as low a dose as 0.5 mmol/L, significantly decreased TG synthesis; the maximal effect was noted at the 3-mmol/L dose (Figure 6A: incorporation, cpm/mg cell protein×10³: using ³H-glycerol, control=149.7±2.1, 0.5 mmol/L niacin=134.9±4.5, 3 mmol/L niacin=102.5±5.4; Figure 5A: using ¹⁴C-acetate, control=208.8±7.1, 0.5 mmol/L niacin=194.2±2.7, 3 mmol/L niacin=149.5±15.2). Because niacin inhibited the synthesis of both fatty acids and TG, we further examined whether niacin has a significant role in the esterification of fatty acids to form TG. These studies were performed by examining the incorporation of radiolabeled oleic acid into TG. The results indicated that the incubation of HepG2 cells with niacin significantly inhibited (10% to 15%) oleic acid esterification to form TG (Figure 6B). The incubation of niacin at various concentrations (0 to 3 mmol/L) did not significantly alter the synthesis of cholesterol and cholesteryl esters as assessed by ¹⁴C-acetate incorporation into newly synthesized cholesterol and cholesteryl ester (¹⁴C-acetate incorporation, cpm/mg cell protein×10³: in cholesterol, control=105.6±7.8, 1 mmol/L niacin=102.8±7.2, 3 mmol/L niacin=99.7±5.2; in cholesteryl ester, control=8.0±0.13, 1 mmol/L niacin=7.7±0.25, 3 mmol/L niacin=7.8±0.48). Similarly, niacin had no effect on the incorporation of

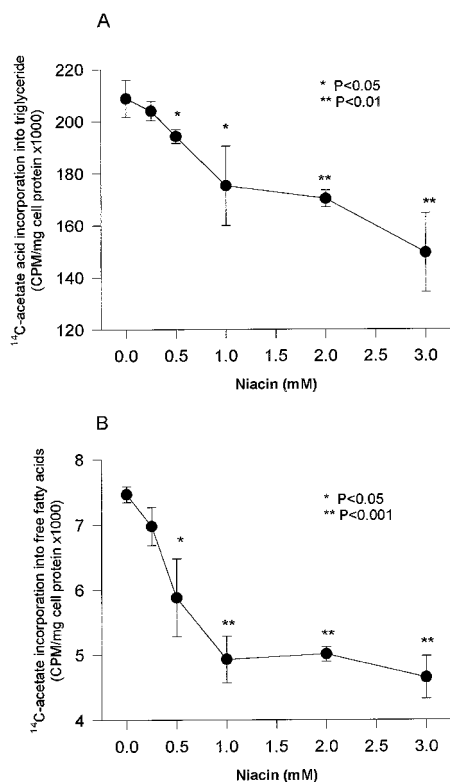


Figure 5. Effect of niacin on the incorporation of radiolabeled acetic acid into newly synthesized TG (A) and fatty acids (B). HepG2 cells were preincubated for 48 hours with varying concentrations of niacin (0 to 3.0 mmol/L). The cells were washed twice with serum-free DMEM and incubated at 37°C in the same medium containing ¹⁴C-acetic acid (1 μCi/mL) and same concentrations of niacin for 4 hours. After incubation, medium was removed and cells were washed thoroughly with PBS. Total lipids were extracted by chloroform/methanol (2:1, vol/vol), dried under nitrogen and reconstituted in 100 μL hexane. Radiolabeled lipids were separated by subjecting an aliquot of total lipid extract to thin-layer chromatography on silica-coated plates using hexane/diethylether/acetic acid (80:20:1 vol/vol/vol) as a developing solvent. Individual lipid bands were visualized by exposure to iodine vapors and identified by comparison with authentic standards. Bands corresponding to triglycerides and fatty acids were scraped and radioactivity measured by liquid scintillation and expressed as CPM/mg cell protein. Data are mean ± SE of 3 separate experiments done in triplicate. Statistical significance was compared with results of controls.

Because MTP, a carrier protein of lipids from their site of synthesis to nascent lipoproteins within the ER, has been implicated in intracellular apoB processing, additional studies were carried out to examine whether niacin-induced apoB degradation is mediated through alterations in MTP activity in HepG2 cells. The incubation of HepG2 cells with varying concentrations of niacin (0 to 3 mmol/L) did not significantly alter MTP activity when compared with controls (MTP activity, nanomoles triacylglycerol transferred per hour per milligram protein: control = 371 ± 15, niacin at 1-, 2-, and 3-mmol/L doses = 371 ± 11, 367 ± 2.4, and 374 ± 6.9, respectively).

In addition to the role of niacin in intracellular apoB degradation, further experiments were planned to examine the effect of niacin on the steady-state mRNA expression of apoB and the uptake of radiolabeled apoB by HepG2 cells to determine the role of niacin on apoB synthesis and uptake by cells. The incubation of HepG2 cells with niacin (0 to 3 mmol/L) for 48 hours did not significantly alter the

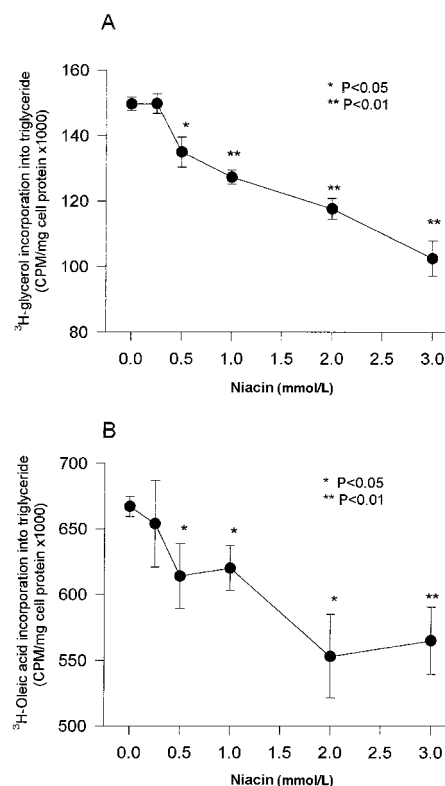


Figure 6. Effect of niacin on the incorporation of ³H-glycerol into TG (A) and the incorporation of ³H-oleic acid into TG (B). HepG2 cells were preincubated for 48 hours with varying concentrations of niacin (0 to 3.0 mmol/L). The cells were washed twice with serum-free DMEM and incubated at 37°C in the same medium containing either ³H-glycerol (5 μCi/mL) or ³H-oleic acid (1 μCi/mL) and same concentrations of niacin for 4 hours. After incubation, cellular lipids were extracted, TGs separated by TLC and the radioactivity measured in TG bands as described in Figure 4. Data are mean ± SE of 3 separate experiments done in triplicate. Statistical significance was compared with results of controls.

steady-state mRNA expression of apoB when compared with controls. Quantitative analysis by densitometric scanning of apoB mRNA blots and normalization with GAPDH message were performed. The densitometric arbitrary values of apoB mRNA expression for control and 0.5- to 3-mmol/L niacin-treated cells were 4.22 and 4.23 to 4.34, respectively. Additional studies were carried out to examine the effect of niacin on the uptake of radiolabeled LDL by HepG2 cells. Uptake studies were performed by incubating control or niacin-pretreated HepG2 cells (48 hours) with ¹²⁵I-LDL for 6 hours. The results from these studies showed that the preincubation of HepG2 cells with niacin (0 to 2 mmol/L) for 48 hours did not significantly alter the uptake of ¹²⁵I-LDL as compared with the respective controls (¹²⁵I-LDL uptake, cpm/mg cell protein: control = 40 571 ± 322, niacin-treated cells at doses of 0.5, 1.0, and 2.0 mmol/L = 40 559 ± 1926, 37 947 ± 1502, and 41 319 ± 3254, respectively).

Discussion

Niacin is widely used to regulate abnormalities in plasma lipid metabolism and in the treatment of atherosclerotic cardiovascular disease. In pharmacologic doses, niacin reduces total plasma cholesterol, triglycerides, VLDL and LDL and increases HDL. Furthermore, various clinical tri-

als, including the Coronary Drug Project, the Cholesterol-Lowering Atherosclerosis Study and the Familial Atherosclerosis Treatment Study, indicated that treatment with niacin significantly reduced total mortality and coronary events and retarded the progression of coronary atherosclerosis.¹⁵⁻¹⁷ Although these studies clearly indicated the beneficial effects of niacin in the treatment of atherosclerotic cardiovascular disease, the cellular and molecular mechanisms by which niacin exerts its effect on lipoprotein metabolism is not clearly defined. Using HepG2 cells as an *in vitro* model, we have recently reported that niacin selectively decreases hepatic removal of HDL apoAI but not cholesteryl esters, suggesting a new concept with regard to the mechanism by which niacin raises HDL.²⁸ However, the hepatocellular mechanisms of niacin in reducing apoB-containing lipoproteins are largely obscure. To address this question, we have performed a series of studies examining the role of niacin on intracellular apoB degradation and key regulatory mechanisms that govern hepatic post-translational processing of apoB and its secretion in HepG2 cells.

The results presented in this report indicate that niacin significantly increased intracellular apoB degradation as assessed by pulse-chase experiments. During a 1-hour chase period, niacin at 0.5 to 3 mmol/L concentration retained less intracellular radiolabeled apoB by 31.5% to 42% as compared with control cells. Similarly, niacin treatment resulted in a lowered amount of radiolabeled apoB secreted into the culture media by 18% to 24% when compared with controls (Table), strongly indicating enhanced apoB degradation as 1 possible mechanism. This increase in apoB degradation by niacin was further demonstrated by varying the chase period from 10 to 180 minutes and showed cellular apoB degradation of 25.5% to 72.2% when compared with an apoB degradative rate of 19.4% to 56.8% in control cells. Because cellular radiolabeled apoB measured by immunoprecipitation techniques may give rise to a nonspecific artifact, we further analyzed the amount of cellular radiolabeled full-length or degraded apoB by SDS-PAGE and fluorography in control and niacin-treated cells at various chase-time points. Similar to the data obtained by immunoprecipitation techniques, SDS-PAGE analysis also showed comparable increases in apoB degradation in cells treated with niacin when compared with controls, and thus validated the use of immunoprecipitation techniques to measure apoB degradation in all of the remaining studies presented in this investigation. Although niacin treatment showed lower signals for full-length apoB bands as compared with controls, we could not detect any degraded apoB bands (ie, smaller molecular weight bands) in either control or niacin-treated cells. This may be due to various factors; the degraded apoB fragments may not be recognized by anti-apoB used for immunoprecipitation or the signals for the degraded products may be too weak to be detected on the fluorogram.

Although the detailed intracellular processes that lead to the assembly of apoB-containing lipoproteins in or within the ER and its subsequent secretion are not fully described, available data suggest that protease(s) localized within the cytosol of the ER may play a crucial role in apoB degradation before its secretion.²³ Furthermore, the synthesis and association of core lipids (eg, triglycerides and cholesteryl esters) has been proposed to protect newly synthesized apoB from

intracellular degradation,^{8,9} suggesting that newly synthesized lipids play an important role in targeting apoB for secretion. Earlier studies used ALLN (calpain inhibitor I), a potent inhibitor of the degradation of both 3-hydroxy-3-methylglutaryl-coenzyme A and the T-cell receptor α subunit in the ER, to determine the role of certain protease-mediated pathways in intracellular apoB degradation.^{23,29,30} It has been shown that the treatment of HepG2 cells with ALLN markedly decreased intracellular apoB degradation and thus may serve as an important tool to understand ALLN-inhibitable protease-dependent apoB degradation in response to various agonists.

In this study, using ALLN, we examined whether niacin-induced apoB degradation is dependent or independent of ALLN-inhibitable protease-mediated pathways. The incubation of HepG2 cells with ALLN markedly inhibited intracellular apoB degradation when compared with control cells, an observation similar to those reported in earlier studies using HepG2 cells.^{6,23} Addition of ALLN to either control cells or niacin-treated cells showed comparable degrees of apoB degradation as compared with their respective controls. Because of the similar percent reduction of apoB degradation by ALLN in control and niacin-treated cells, these results suggest that the effect of niacin to induce apoB degradation is independent of ALLN-inhibitable protease-mediated pathways. These observations also rule out the possibility of other putative proteases that may be sensitive to niacin stimulation because decreased ALLN-induced apoB degradation was unaffected by niacin.

Additional studies were designed to determine whether alterations in the association of newly synthesized triglycerides with apoB are involved in niacin-induced cellular apoB degradation. To explore this possibility, we have used oleic acid, a known agonist that has been employed to decrease intracellular apoB degradation. Earlier studies have indicated that the treatment of HepG2 cells with oleic acid markedly inhibited intracellular apoB degradation with a parallel increase in apoB secretion into culture media without altering mRNA expression of apoB.⁷⁻⁹ Furthermore, Boren et al⁷ suggested that the cotranslational addition of lipids (eg, triglyceride) was important for the rapid transfer of apoB to the ER lumen and subsequent secretion. Using acetyl coenzyme A carboxylase inhibitor and HMG-CoA reductase inhibitor, agents to block fatty acid and cholesterol synthesis, respectively, recent studies have indicated that the rate of triglyceride but not cholesterol synthesis regulates apoB degradation in HepG2 cells.³¹ Furthermore, these authors also showed that the rate of triglyceride synthesis rather than the total intracellular mass of triglycerides determines the secretion of apoB. Because the mobilization of stored lipid droplets for VLDL assembly is impaired in HepG2 cells,³² Benoist and Grand-Perret suggested that neutral lipid synthesis is compartmentalized and that the triglycerides available for lipidation of apoB and its subsequent secretion are synthesized in the proximity of lipoprotein assembly sites, whereas stored triglycerides are not directly available for the assembly of apoB-containing lipoproteins and subsequent secretion.³¹ Sakata et al,²³ using a combination of the ALLN and oleate approaches, proposed that oleate treatment, by increasing triglyceride synthesis, reduces degradation of

apoB by accelerating its translocation from the protease compartment of the ER membrane.

In this investigation, cellular apoB degradation in HepG2 cells treated with niacin plus oleic acid was significantly higher than in cells treated with oleic acid alone. This difference in apoB degradation was significant at various chase-time points from 10 to 60 minutes. Because oleate-mediated increased TG synthesis did not protect against niacin-induced apoB degradation, we hypothesized that niacin, through alterations in TG synthesis, may increase apoB degradation. To confirm this possibility, we examined the direct effects of niacin on the synthesis of TG, cholesterol, cholesteryl ester, and phospholipids by measuring the incorporation of ^3H -glycerol or ^{14}C -acetate into corresponding lipids. Results showed that the incubation of HepG2 cells with niacin significantly inhibited TG synthesis by 20% to 40% as determined by radiolabeled acetate or glycerol incorporation, respectively. Because TG synthesis is regulated by cellular processes involved in fatty acid synthesis and their esterification to form TG, we examined the effect of niacin on fatty acid synthesis and on the esterification of fatty acids to produce TG. Niacin significantly inhibited the synthesis of fatty acids and the esterification of fatty acids to synthesize TG. Thus, the data indicate that niacin inhibits TG production at 2 synthetic sites: (a) fatty acid synthesis from acetate, and (b) esterification of fatty acids to form TG. Because these 2 cellular processes are regulated by various enzyme systems and esterification reactions, niacin may have yet unknown roles in modulating these processes, which are outside the scope of this investigation. In contrast to triglyceride synthesis, niacin had no effect on the synthesis of cholesterol or cholesteryl esters in HepG2 cells. Our *in vitro* findings on triglyceride synthesis are in line with earlier *in vivo* studies in humans by Grundy et al.¹³ demonstrating that the treatment with niacin reduced VLDL-triglyceride synthetic rates (transport) as determined by multi-compartmental kinetic analysis after the injection of radiolabeled glycerol as a precursor. It has been suggested that the observed reduced influx of VLDL-triglycerides in niacin-treated patients may be associated with the ability of niacin to inhibit the release of free fatty acids by adipose tissue.^{33–35} The quantitative roles of the effect of niacin on decreased fatty acid mobilization versus decreased hepatic triglyceride synthesis is unclear. Because niacin decreases triglyceride content in VLDL, it is possible that such triglyceride-poor particles may result in smaller and denser VLDL particles as suggested by kinetic studies.¹³ Increased hepatocyte apoB degradation by niacin would also decrease the number of VLDL (and their catabolic product, LDL) particles secreted, and explain the lower apoB concentrations observed clinically after niacin treatment.¹⁷

Thus, we propose that niacin, by inhibiting triglyceride synthesis through multiple mechanisms, may hinder the lipidation and translocation of apoB across the ER membrane and may create a favorable environment for protease-mediated intracellular apoB degradation. The inability of niacin to alter cholesterol or cholesteryl ester synthesis is congruent with previous studies indicating that apoB degradation is not dependent on cholesteryl ester synthesis.^{31,36} In addition to the synthesis of triglyceride, MTP, a protein that catalyzes the transfer of triglycerides, cholesteryl esters and phosphatidylcholine between membranes, has been recently

shown to influence apoB degradation and secretion.^{10–12} The treatment of HepG2 cells with niacin did not alter MTP activity, suggesting that the effect of niacin on apoB degradation is independent of MTP-mediated pathways.

In addition to the effects of niacin on apoB degradation, decreased apoB secretion in niacin-treated cells may be the result of alterations in apoB mRNA expression and synthesis, and the uptake of apoB-containing lipoproteins by HepG2 cells. Because niacin did not alter either apoB mRNA expression or radiolabeled LDL uptake, we rule out the possibility that changes in apoB gene expression or protein uptake (via LDL receptor) contributed to the decreased apoB. Data presented in this communication indicate that niacin is directly responsible for the effects observed. Although it would be important to make some correlation of *in vitro* niacin concentration used in our studies to those of plasma concentrations of niacin in patients, we feel that it would be difficult to make such correlations based on the following points. The pharmacokinetics of niacin have not been clearly established in humans. However, it has been noted that at pharmacologic doses (>1 g/day) niacin is almost completely absorbed and reaches peak plasma concentrations of 0.12 to 0.24 mmol/L within 30 to 60 minutes. It has also been noted that nicotinic acid is the major metabolite of niacin. After absorption, most of the niacin is taken up in the liver and very little enters the systemic circulation. Thus, plasma levels are not reflective of biological activity. The exact concentration of niacin in the hepatic interstitial compartment has not been established. However, our *in vitro* concentrations of a 10-fold higher order of concentration than plasma concentrations achieved clinically are not out of line. The possibility also exists that niacin and/or its metabolites (eg, nicotinic acid) may participate in these effects. Additional research is needed to elucidate this question.

In summary, these results suggest that niacin, by increasing hepatic post-translational intracellular degradation of apoB, but not altering mRNA expression or uptake, exerts its action to lower the secretion of apoB-containing lipoproteins. Furthermore, the selective inhibition of triglyceride synthesis by niacin may limit the lipidation of apoB required for its translocation across the ER membrane and thus facilitate intracellular apoB degradation, suggesting an explanation for reductions in apoB-containing lipoproteins observed clinically after niacin treatment.

Acknowledgments

This investigation was supported in part by a grant from Kos Pharmaceuticals and by the Long Beach Research Foundation. We thank Noah Wagner for his expert assistance in typing this manuscript and Mark Hubbard for preparation of figures.

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