Niacin noncompetitively inhibits DGAT2 but not DGAT1 activity in HepG2 cells[1]

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Abstract **Niacin is a widely used lipid-regulating agent in dyslipidemic patients. Previously, we have shown that niacin inhibits triacylglycerol synthesis. In this report, using HepG2 cells, we have examined the effect of niacin on the mRNA expression and microsomal activity of diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT2), the last committed but distinctly different enzymes for triglyceride synthesis. Addition of niacin to the DGAT assay reaction mixture dose-dependently (0–3 mM) inhibited DGAT activity by 35–50%, and the IC_{50} was found to be 0.1 mM. Enzyme kinetic studies showed apparent K_m values of 8.3 μM and 100 μM using [^{14}C]oleoyl-CoA and *sn*-1,2-dioleoylglycerol as substrates, respectively. A decrease in apparent V_{max} was observed with niacin, whereas the apparent K_m remained constant. A Lineweaver-Burk plot of DGAT inhibition by niacin showed a noncompetitive type of inhibition. Niacin selectively inhibited DGAT2 but not DGAT1 activity. Niacin inhibited overt DGAT activity. Niacin had no effect on the expression of DGAT1 and DGAT2 mRNA. These data suggest that niacin directly and noncompetitively inhibits DGAT2 but not DGAT1, resulting in decreased triglyceride synthesis and hepatic atherogenic lipoprotein secretion, thus indicating a major target site for its mechanism of action.**—Ganji, S. H., S. Tavintharan, D. Zhu, Y. Xing, V. S. Kamanna, and M. L. Kashyap. **Niacin noncompetitively inhibits DGAT2 but not DGAT1 activity in HepG2 cells. *J. Lipid Res.* 2004.** 45: **1835–1845.**

Supplementary key words triglyceride • lipoproteins • hepatocytes • diacylglycerol acyltransferase

Niacin is an effective, unique lipid-regulating agent that beneficially reduces plasma triglycerides (TGs), cholesterol, and atherogenic apolipoprotein B (apoB)-containing lipoproteins (VLDL, LDL, and lipoprotein [a]) and increases antiatherogenic apoA-I-containing HDL levels (1–3). Several clinical trials have demonstrated that treatment with niacin significantly reduces total mortality and coronary events and retards the progression or induces the regression of coronary atherosclerosis (4–6). Despite its wide usage as a broad-spectrum lipid-regulating agent, the cellular and molecular mechanisms by which niacin modulates the hepatic lipid metabolism and the production of VLDL and LDL particles are incompletely understood.

Using HepG2 cells as an in vitro model system, we have previously shown that niacin inhibited the incorporation of radiolabeled oleic acid or glycerol into TGs, suggesting decreased de novo synthesis of TGs by niacin (7). Additionally, we have shown that niacin also increased intracellular apoB degradation in HepG2 cells (7). Because the synthesis and availability of TG play a critical role in intracellular apoB processing and secretion of apoB-containing lipoproteins (8–10), our previous studies suggested that niacin, by inhibiting TG synthesis, increased intracellular apoB degradation, resulting in reduced secretion of apoB-containing particles (7). In support of these in vitro studies, earlier turnover studies in humans suggested that niacin decreased the production (transport) rate of TG from radiolabeled fatty acids, thus decreasing TG-rich lipoproteins (e.g., VLDL) and their product LDL (11).

Modulation in the TG synthetic pathway and associated key enzyme systems may provide important tools to alter the apoB degradative/secretory pathway involved in hepatic VLDL/LDL secretion and subsequent plasma levels of these lipoproteins. The formation of TG is catalyzed by various enzyme systems associated with the synthesis of fatty acids and the acylation of fatty acids to form TGs. Acyl CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20),

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a microsomal enzyme, plays a central role in the esterification of fatty acids to form TGs (12). DGAT catalyzes the terminal and only committed step in cellular TG synthesis by using diacylglycerol and fatty acyl-CoA as its substrates. The direct biological significance of DGAT in intracellular apoB processing and the secretion of apoB-containing lipoproteins was recently demonstrated in preliminary studies using a rat hepatoma cell line (McA-RH7777) in which the overexpression of human DGAT increased the synthesis of TGs coupled with increased secretion of apoB-containing lipoproteins (13). This molecular approach directly demonstrated the biological significance of DGAT in TG synthesis-mediated effects on intracellular apoB processing and subsequent VLDL/LDL assembly and secretion. Recently, two distinct DGAT1 and DGAT2 genes from two different gene families were identified in mammalian cells, and both DGAT genes share similar specific activity with respect to substrates for TG synthesis (12, 14, 15).

Owen, Corstorphine, and Zammit (16) have recently identified two functional DGAT activities in liver microsomes: *a*) an "overt" DGAT activity associated with the cytosolic aspect of the endoplasmic reticulum (ER) membrane; and *b*) a "latent" DGAT activity associated with the luminal aspect of the ER membrane. Overt DGAT activity was proposed to be involved in the synthesis of TGs of cytoplasmic lipid droplets, whereas latent DGAT activity was suggested to be involved in the synthesis of luminal side TGs destined for assembly into nascent VLDL particles [reviewed in ref. (17)]. In view of these two DGAT activities, Yang et al. (18) have shown that important differences exist between the stereospecific distribution of the fatty acyl moieties in TGs associated with the cytosolic droplet and VLDL TGs. TGs in cytosolic droplet are hydrolyzed to diacylglycerol (DAG) and reesterified before the lipidation of apoB. This reesterification of modified DAG is brought about by overt DGAT, which is located on the cytosolic side of the ER membrane (16). Furthermore, the DAG that is permeabilized through the ER membrane is reesterified by latent DGAT enzyme, which is located in the lumen of the ER (16). Despite these suggestions, further experimental evidence and confirmatory studies are required to clearly understand the contribution of overt and/or latent DGAT activity-mediated TG synthesis in the regulation of intracellular apoB processing and VLDL/LDL assembly and secretion. It is also not known whether the modulation of DGAT activity may play a role in niacin-mediated effects on hepatocyte TG synthesis and associated VLDL/LDL secretion.

In this report, we have hypothesized that niacin may affect DGAT activity in hepatocytes. Studies were designed to examine the direct effect of niacin on the mRNA expression and microsomal DGAT1 and DGAT2 enzyme activities in HepG2 cells. To understand the mode of action of niacin, additional studies were undertaken to define the DGAT enzyme kinetic parameters and the type of enzyme inhibition by niacin. Studies were also carried out to assess whether niacin inhibited overt or latent DGAT activity. The data presented here demonstrate that niacin noncompetitively inhibits microsomal DGAT2 activity without altering DGAT (1 and 2) mRNA expression in HepG2 cells.

MATERIALS AND METHODS

Materials

Tissue culture materials, media, FBS, BSA (essentially fatty acid-free), oleoyl-CoA, *sn*-1,2-dioleoylglycerol, sucrose, 2-propanol, heptane, and niacin were obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]oleoyl-CoA was purchased from Amersham Corporation (Arlington Heights, IL). The HepG2 cell line was obtained from the American Type Culture Collection (Rockville, MD). Bicinchoninic acid Protein Assay Reagent was from Pierce (Rockford, IL). The RNeasy mini kit was from Qiagen. Niacin stock solution was prepared either in 10 mM Tris-HCl buffer or in DMEM culture medium. The pH was adjusted to 7.4 with 1 N NaOH. Appropriate concentrations of niacin were added either directly to the reaction mixture containing microsomes of the DGAT assay system or to the culture flasks for appropriate incubation periods with HepG2 cells as indicated in specific studies. All other chemicals used were of analytical grade.

HepG2 cell culture and treatment

HepG2 cells were grown in 100 mm petri dishes with 12 ml of high-glucose DMEM containing 10% FBS, 2 mmol/l glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. Subcultures were made from confluent stock cultures by trypsinization with 0.25% trypsin-EDTA solution. For isolation of RNA, HepG2 cells were incubated for 6, 24, and 48 h in the absence or presence of niacin (3.0 mmol/l) in DMEM containing 10% FBS. 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 (3 mmol/l) had no effect on cell viability. During the incubation period of 48 h, ~1–2% of the cells were stained with trypan blue in both control and 1–3 mmol/l niacin-treated cells. Additionally, no significant differences in DNA or protein contents were observed between control and niacin-treated cells.

Preparation of microsomes from HepG2 cells

The microsomal fractions from HepG2 cells were prepared as described previously (19–21). Briefly, 70–80% confluent HepG2 cells were washed twice with cold PBS and were collected in 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA (STE buffer) by scraping under cold conditions (4°C) and were homogenized with Branson Sonifier 450. The homogenate was centrifuged at 25,000 *g* for 15 min at 4°C. The supernatant was then centrifuged at 100,000 *g* for 1 h at 4°C to obtain the microsomal pellet. Microsomes were resuspended in STE buffer and stored at −70°C and used for DGAT assay within 4 weeks. The protein concentration of the microsomes was determined by the bicinchoninic acid method.

The purity of the microsomal fractions was assessed by measuring NADPH-cytochrome *c* reductase activity as a marker for the microsomal fractions by using an assay kit (Sigma). The NADPH-cytochrome *c* reductase activities (units per milligram of protein) in microsomal and cytosolic fractions were 0.90 and 0.25, respectively. The enrichment of NADPH-cytochrome *c* reductase activity (~80%) in the microsomal fractions indicates the purity of the microsomal fractions used for the studies. Mitochondrial contamination in the microsomal fractions was checked by measuring succinate-cytochrome *c* reductase activity (a marker

for mitochondria) as described previously (22). In microsomal preparations, the succinate-cytochrome *c* reductase activity was not detected, suggesting that there was no contamination of mitochondria in microsomal preparations.

Measurement of total DGAT activity

The DGAT activity in the microsomal fractions was measured by previously described procedures using [^{14}C]oleoyl-CoA and *sn*-1,2-dioleoylglycerol as substrates (20). Initially, we optimized DGAT assay conditions with respect to incubation time, microsomal protein concentration, and pH of the assay reaction mixture. Optimal assay conditions were used for the DGAT assay in the absence or presence of niacin. In brief, 200 μl of reaction mixture contained 200 μM *sn*-1,2-dioleoylglycerol in acetone, 30 μM [^{14}C]oleoyl-CoA, 8 mM $MgCl_2$, 200 μg of BSA, and 10 μg of microsomal protein in 175 mM Tris-HCl buffer, pH 8.0. A measured amount of niacin was added to the assay mixture. The reaction mixture was incubated for 30 min at 25°C. After incubation, the reaction was stopped by the addition of 1.5 ml of stop solution [2-propanol-heptane-water (80:20:2, v/v)]. The top heptane phase was collected and washed with alkaline ethanol. An aliquot of the top heptane phase was used for radioactivity measurements using a Beckman LS 5801 counter. The DGAT activity was expressed as nanomoles of TG formed per minute per milligram of microsomal protein.

Measurement of DGAT1 and DGAT2 activity

Earlier studies have shown that DGAT2 activity is inhibited at higher concentrations of $MgCl_2$ (100 mM) (14). To determine whether niacin inhibits DGAT1 or DGAT2 activity, the DGAT assay was carried out in the presence of 8 mM and 100 mM $MgCl_2$. DGAT2 activity was calculated by subtracting the DGAT activity value obtained at 100 mM $MgCl_2$ from the value obtained at 8 mM $MgCl_2$.

Measurement of overt and latent DGAT activity

Overt DGAT activity in HepG2 cell microsomes was measured by carrying out the DGAT assay using unpermeabilized microsomes. Latent DGAT activity was measured in microsomes after permeabilization of microsomal vesicles with alamethicin (16). Microsomes (2 mg protein/ml) were incubated for 30 min on ice with 0.04 mg alamethicin/ml in 0.38% (v/v) ethanol for permeabilization. Mannose-6-phosphatase activity was measured in parallel with the DGAT activity in permeabilized and unpermeabilized microsomes. Mannose-6-phosphatase, an established latent enzyme in microsomes, served as a marker for determining the leakiness of the microsomal preparations (16). The assay buffer for mannose-6-phosphatase activity consisted of 50 mM sodium cacodylate, pH 6.6, 0.2 M sucrose, 2.5 mM dithiothreitol, 25 mM mannose-6-phosphate, and 10 mg/ml defatted BSA. Thirty microliters of this reaction mixture was incubated with 150 μl of microsomal protein at 37°C for 10 min. The reaction was stopped by the addition of 125 μl of 20% (v/v) perchloric acid. Inorganic phosphorus generated was quantified in the supernatant using the Sigma Pi determination kit.

Using DGAT activity measured in the absence or presence of alamethicin coupled with correction for the nonlatency of mannose-6-phosphatase and membrane leakiness, the following equation was used to calculate overt and latent DGAT activity (16, 17): overt DGAT = $D_0 - [(D_t - D_0)M_0/M_t]$, and latent DGAT = $(D_t - D_0)M_t/(M_t - M_0)$, where D_0 and D_t represent DGAT activity before and after treatment with alamethicin, respectively, and M_0 and M_t represent mannose-6-phosphatase activity before and after treatment with alamethicin, respectively. The mannose-6-phosphatase activities in microsomal preparations before and after alamethicin treatment were found to be 78.61 and 164.31 nmol Pi formed/min/mg protein, respectively.

Measurement of monoacylglycerol acyltransferase activity

Monoacylglycerol acyltransferase (MGAT) activity was determined as described previously (21). In brief, 200 μl of reaction mixture contained 175 mM Tris-HCl, pH 7, 1 mg/ml BSA, 15 μg of a 1:1 (w/w) mixture of phosphatidylcholine and phosphatidylserine sonicated in 10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 25 μM [^{14}C]oleyl-CoA, and 50 μM 2-monooleylglycerol dispersed in acetone (concentration less than 2.5%). The reaction was initiated by adding 50 μg of microsomal protein and was incubated at 25°C for 30 min. The products were extracted into heptane and washed twice with alkaline ethanol. An aliquot of the final heptane phase was used for radioactivity counting. The remaining heptane phase was concentrated and applied to TLC using heptane-isopropyl ether-acetic acid (60:40:4, v/v) as a solvent system. Lipids were visualized by exposure to iodine vapors. The spots corresponding to DAG and triacylglycerol were scraped and counted. MGAT specific activity was calculated by subtracting one-half of the counts that appeared in triacylglycerol.

Semiquantitative RT-PCR analysis

The cells were grown in 100 mm culture Petri dishes in 10 ml of DMEM for 3 days until they attained 60–70% confluence. To study the effect of niacin on the expression of DGAT mRNA, cells were treated with 3.0 mM niacin for 6, 24, and 48 h. Cells were collected by trypsinization, and total RNAs were extracted using the RNeasy mini kit (Qiagen). The isolated RNA was subjected to RNase-free DNase-I digestion to remove DNA contamination. cDNA was synthesized from 850 ng of total RNA in 20 μl using random hexamers and murine Moloney leukemia virus reverse transcriptase (Life Technologies, Inc.). The DGAT1 primer was designed using BLAST database search for the human DGAT gene. The sense primer was 5′-GGCCTTCTTCCACGAGTACC-3′ and the antisense primer was 5′-GGCCTCATAGTTGAGCACG-3′. Sense and antisense primers for DGAT2 were same as those reported previously: sense primer, 5′-AGTGGCAATGCTATCATCAT-3′; antisense primer, 5′-GAGGCCTCGACCATGGAAGAT-3′ (23). Semiquantitative RT-PCR analysis was performed starting with first-strand cDNA from reverse transcription with 150 nM sense and antisense primer in a final volume of 50 μl. Sense and antisense primers for GAPDH (150 nM) were also included in the reaction mixture. PCR amplification products at different cycles were separated on a 1.2% agarose gel and were visualized with ethidium bromide. The band intensity was measured using the EagleSight gel documentation system.

Statistical analysis

The data presented are means ± SEM of three experiments performed at least in duplicate. Statistical significance was calculated by using Student's *t*-test, and $P < 0.05$ was considered significant.

RESULTS

Effect of niacin on DGAT activity and IC_{50}

Initial enzyme assay optimization studies were performed to determine the optimal conditions, including incubation period, microsomal protein concentrations, and pH, for the measurement of DGAT activity in HepG2 cell microsomes. The data showed that the DGAT activity increased linearly with respect to the increased incubation

periods between 15 and 60 min at 25°C. DGAT activities (nmol TG formed/mg protein) at various incubation periods were as follows: 15 min = 48.62 ± 1.23, 30 min = 76.18 ± 2.01, 45 min = 115.04 ± 4.36, and 60 min = 132.93 ± 4.45. Additional data indicated that the DGAT activity increased linearly with increases in microsomal protein concentration between 5 and 20 μg. DGAT activities (nmol TG formed/min) with varying microsomal protein concentrations were as follows: 5 μg = 0.015 ± 0.001, 10 μg = 0.023 ± 0.001, 15 μg = 0.032 ± 0.002, and 20 μg = 0.038 ± 0.004. The optimal pH for the DGAT assay system was determined by measuring enzyme activity in the presence of Tris-HCl buffer with pH in the range of 7–9. The data indicated that the pH optimum for DGAT activity in HepG2 cell microsomes was 8.0 (data not shown). In studies assessing the effect of niacin on DGAT activity, we used optimal conditions of 30 min of incubation and 10 μg of microsomal protein that are within the linear assay range.

To determine the direct effect of niacin on DGAT activity, DGAT activity was measured by adding niacin directly to the DGAT assay reaction mixture containing HepG2 cell microsomal fraction and [^{14}C]oleoyl-CoA and DAG as substrates. The data indicated that the addition of niacin (0–3 mM) to the assay reaction mixture dose-dependently inhibited microsomal DGAT activity. The dose-response curve of DGAT inhibition showed that niacin as low as 0.05 mM significantly inhibited DGAT activity, and the maximal inhibition was noted at 1 mM and remained nonsignificantly different at 3 mM niacin (**Fig. 1**). The percentage inhibition of DGAT activity at the highest niacin concentration (3 mM) in various experiments varied from 35–50% with different batches of microsomal fractions and with the confluence of HepG2 cells used for microsomal isolation. Fig. 1, inset, displays data on the percentage inhibition of DGAT activity as a function of various concentrations of niacin. The calculated IC_{50} of niacin to inhibit DGAT activity was found to be 0.1 mM.

DGAT enzyme kinetic parameters and the nature of enzyme inhibition by niacin

Because DGAT uses both oleoyl-CoA and DAG as substrates, we performed two sets of experiments using these substrates to determine DGAT enzyme kinetic parameters, including apparent K_m, apparent V_{max}, dissociation constant of the enzyme-inhibitor complex (K_i), and the type of enzyme inhibition. In the first set, the DGAT assay was carried out by varying the [^{14}C]oleoyl-CoA concentration (2.5–15 μM) and keeping the concentration of *sn*-1,2-dioleoylglycerol (200 μM) constant to determine the reaction velocity and apparent V_{max} in the absence and presence of 0.5 and 3.0 mM niacin. The reaction velocity of DGAT was obtained by plotting the rate of formation of TG as a function of varying concentrations of [^{14}C]oleoyl-CoA (**Fig. 2A**). The results showed that niacin (0.5 and 3 mM) decreased apparent V_{max} by 29–32% compared with the control (Fig. 2A).

The competitive/noncompetitive type of DGAT inhibition by niacin was assessed by plotting the reciprocal of the reaction velocity versus the reciprocal of the substrate oleoyl-CoA concentration [Lineweaver-Burk plot (LB plot); Fig. 2B]. For the LB plot, we have adopted commonly

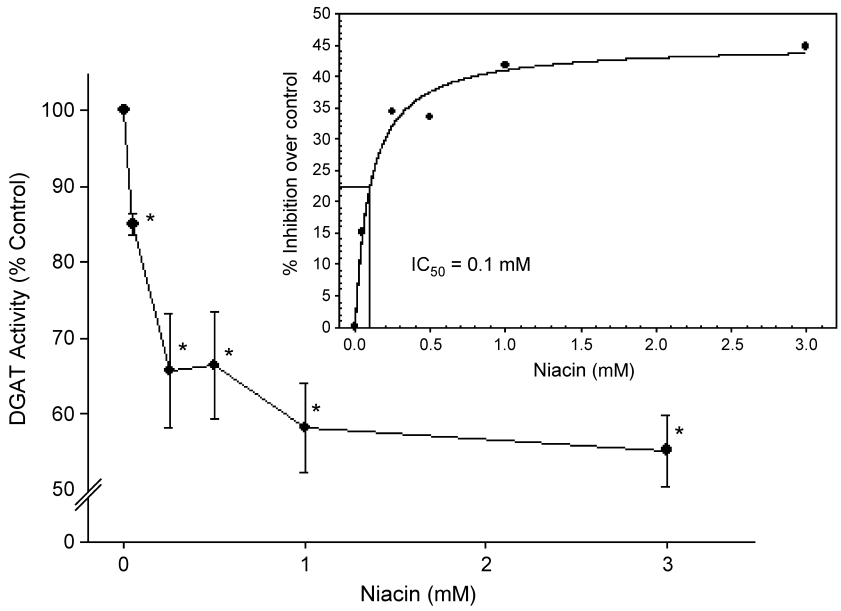

Fig. 1. Dose-response curve of the effect of niacin on diacylglycerol acyltransferase (DGAT) activity. DGAT activity was measured by adding niacin (0–3 mM) directly to the assay reaction mixture containing [1-^{14}C]oleoyl-CoA and *sn*-1,2-dioleoylglycerol as substrates and HepG2 cell microsomal fractions. Data shown are means ± SEM of six experiments, except at 0.05 mM niacin concentration, where n = 3. The inset shows the plot of percentage inhibition of DGAT activity versus niacin concentration. Data used are means from the dose-response curve. * $P < 0.05$ compared with controls.

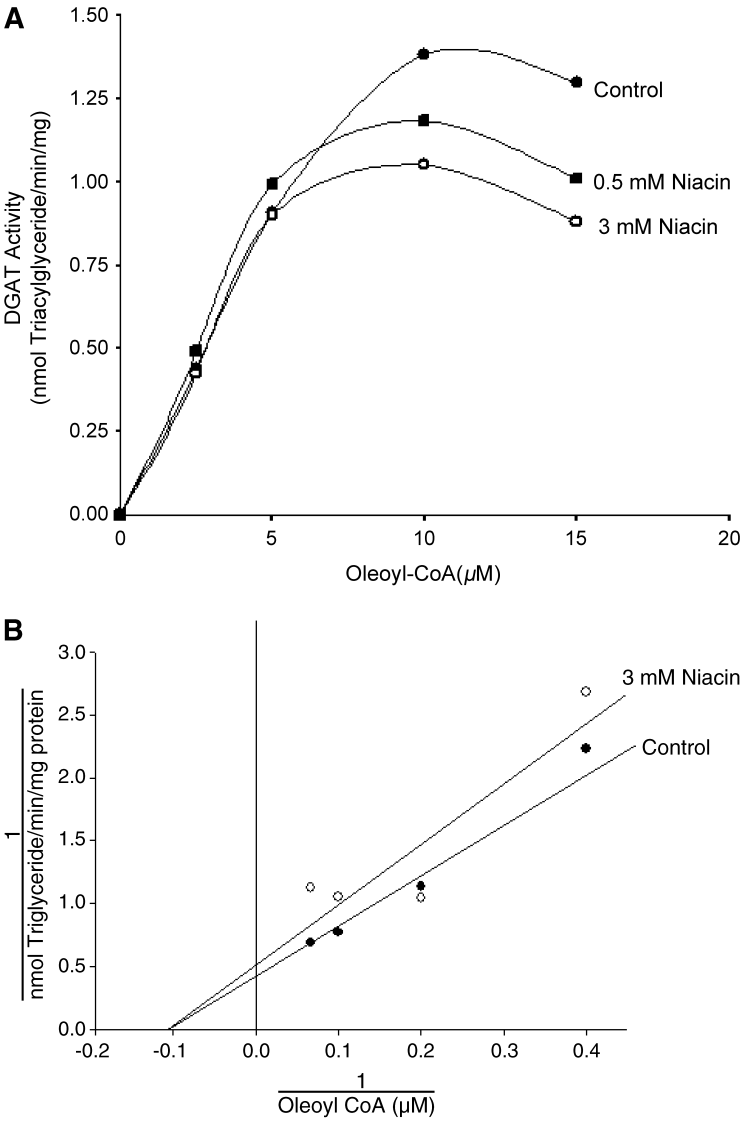

Fig. 2. A: Initial velocity plots showing the dependence of DGAT activity on [1-^{14}C]oleoyl-CoA in the absence and presence of niacin (0.5 and 3.0 mM). DGAT activity was measured at varying concentrations of [1-^{14}C]oleoyl-CoA in the absence and presence of niacin (as described in Materials and Methods). B: Lineweaver-Burk (LB) plot of the inhibition of DGAT. The reciprocal of the rate of formation of triglycerides (TGs) was plotted against the reciprocal of [1-^{14}C]oleoyl-CoA concentration. The data are means of two determinations from a representative experiment. We repeated these experiments three times and obtained similar results.

used standard linear transformation of data and a best-fit line drawn through the data points. Because of the substrate inhibition of DGAT activity (Figs. 2A and 3A), we could use only a few points that are in the linear range in the initial velocity curve. Apparent K_m was calculated based on the intercept on the negative X-axis of the LB plot, and the apparent K_m was found to be 8.3 μM for [^{14}C]oleoyl-CoA as a substrate (Fig. 2B). The apparent V_{max} (for control) and the apparent $V^I_{\max}$ (for 3 mM niacin) were calculated from the intercept on the Y-axis of the LB plot. The data from the LB plot indicated that niacin caused a decrease in apparent V_{max} compared with controls, whereas the apparent K_m remained constant between control and 3 mM niacin (Fig. 2B). The LB plot also showed that niacin inhibition of DGAT follows a noncompetitive pattern of enzyme inhibition (Fig. 2B). The K_i was calculated based on the formula $V^I_{\max} = V_{max}/1 + [I]/K_i$, where [I] is concentration of inhibitor (niacin). Based on this equation, the K_i was found to be 5.57 μM. Although the LB plot is commonly used for kinetic parameter studies, it generally suffers from some errors attributable to skewing of the data when reciprocals are used. For example, the smaller and more error-prone experimental data become the largest values in the plot. For these reasons, some of the data points in Figs. 2B and 3B are not close to the best-fit line drawn in LB plots.

In the second set, the DGAT assay was carried out by varying the DAG concentration (25–200 μM) and keeping the concentration of [^{14}C]oleoyl-CoA (30 μM) constant to determine the reaction velocity and apparent V_{max} in the absence and presence of 3 mM niacin. The rate of formation of TG was plotted against the varying concentrations of DAG (**Fig. 3A**). The results showed that niacin (3 mM) decreased the apparent V_{max} by ~30% at 125 μM DAG concentration, and the inhibitory effect was higher (70%) at 200 μM DAG (Fig. 3A). The apparent K_m and K_i for DAG were calculated from the LB plot (Fig. 3B) and were found to be 100 μM and 6 μM, respectively. Using DAG as a substrate, the LB plot also showed noncompetitive DGAT inhibition by niacin (Fig. 3B).

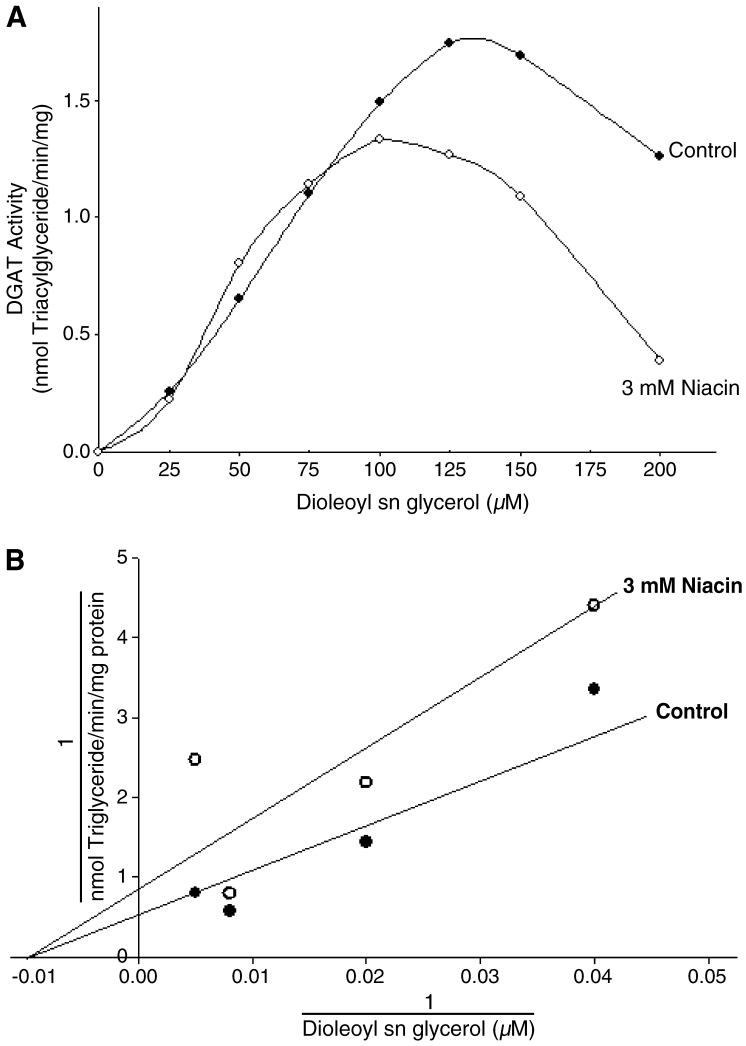

Fig. 3. A: Initial velocity plots showing the dependence of DGAT activity on *sn*-1,2-dioleoylglycerol in the absence and presence of niacin (3.0 mM). DGAT activity was measured at varying concentrations of *sn*-1,2-dioleoylglycerol in the absence and presence of niacin (as described in Materials and Methods). B: LB plot of the inhibition of DGAT. The reciprocal of the rate of formation of TG was plotted against the reciprocal of *sn*-1,2-dioleoylglycerol concentration. The data are means of two determinations from a representative experiment. We repeated these experiments three times and obtained similar results.

Effect of niacin on DGAT1 and DGAT2 activity

Because the acylation of fatty acids to form TGs is catalyzed by two distinct DGATs, DGAT1 and DGAT2, we further examined the effect of niacin on DGAT1 and DGAT2 activity in the presence or absence of high concentrations of $MgCl_2$ (100 mM) to distinguish DGAT1 and DGAT2 activities. Addition of niacin (3 mM) to the DGAT assay mixture completely inhibited DGAT2 activity (**Fig. 4**). In contrast to DGAT2 activity, niacin did not affect DGAT1 activity (Fig. 4). These data clearly indicate that niacin selectively inhibited DGAT2 without affecting DGAT1 activity.

Effect of niacin on overt and latent DGAT activity

DGAT activity was previously shown to exist in the ER membrane cytosol-facing overt and lumen-facing latent forms to catalyze the esterification of fatty acids to form TGs within the cytoplasm and lumen of ER membrane (24). To assess the effect of niacin on overt and latent DGAT activity, we examined DGAT activity in unpermeabilized and permeabilized microsomes. As shown in **Fig. 5**, niacin (3 mM) significantly inhibited overt DGAT activity by 30%. Niacin also decreased latent DGAT activity by 30%, although this effect did not reach statistical significance (Fig. 5).

Effect of niacin on MGAT activity

To determine whether niacin's effects are specific for DGAT or generalized for acyltransferases, we measured MGAT activity as a representative enzyme for acyltransferases involved in the TG synthetic pathway. Low levels of MGAT activity was detected in HepG2 cell microsomes. Addition of niacin (3 mM) to the assay mixture did not inhibit MGAT activity (control = 0.0068 ± 0.00067 and 3 mM niacin = 0.0066 ± 0.00068 nmol DAG formed/min/mg protein).

Effect of niacin on DGAT mRNA expression

Studies were performed to determine the effect of niacin on mRNA levels of DGAT1 and DGAT2 by semiquantitative RT-PCR. HepG2 cells were treated with 3 mM niacin in DMEM containing 10% FBS for 6, 24, and 48 h. A representative RT-PCR blot for DGAT1, DGAT2, and GAPDH in control and niacin-treated cells is shown in **Fig. 6**. The quantitative analysis of DGAT1 and DGAT2 mRNA was assessed by normalizing the DGAT mRNA message (densito-

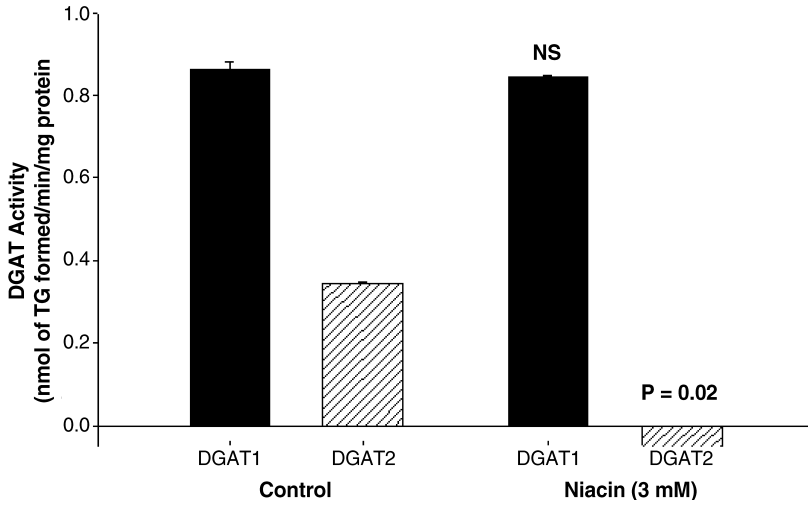

Fig. 4. Effect of niacin on DGAT1 and DGAT2 activity. DGAT1 and DGAT2 activities in HepG2 cell microsomes were measured in the absence or presence of high concentrations of $MgCl_2$ (100 mM) as described in Materials and Methods. The data shown are means ± SEM of three determinations from a representative experiment. Three such experiments were performed, and similar results were obtained. The *P* value indicated refers to DGAT2 activity compared with control.

metric units of band intensity) with the housekeeping enzyme GAPDH message. As shown in **Table 1**, data from three experiments indicated that the treatment of cells with niacin did not affect the mRNA levels of either DGAT1 or DGAT2 compared with controls.

DGAT activity in microsomes isolated from control and niacin-treated cells

HepG2 cells were treated in the absence or presence of niacin (3 mM) for 48 h. Cells were collected and microsomes were isolated for DGAT activity assays. The results indicated that the DGAT activity was similar in microsomes isolated from control or niacin-treated cells. DGAT activities in control and niacin-treated cell microsomes were 3.25 ± 0.18 and 3.22 ± 0.19 nmol TG/min/mg protein.

DISCUSSION

DGAT is the terminal and only committed enzyme that catalyzes the acylation of DAG in the synthesis of TGs (25–28). However, DAG used in the DGAT reaction can be derived from multiple pathways, including *1*) hydrolysis of phosphatidic acid produced by the de novo synthesis pathway from glycerol-3-phosphate catalyzed by glycerol-3-phosphate acyltransferase; *2*) esterification of monoacylglycerol catalyzed by MGAT; and *3*) hydrolysis of TG or phospholipids. Previously, we have shown that niacin inhibits the incorporation of radiolabeled glycerol or oleic acid into cellular TGs (7). This observation led us to hypothesize that niacin may inhibit DGAT, resulting in the inhibition of TG synthesis. The data indicate that addition of niacin to the DGAT assay reaction mixture inhibited microsomal DGAT activity by 35–50%, suggesting a direct effect of niacin on DGAT catalytic domain(s). MGAT (another acyltransferase) activity was measured to determine whether niacin's effects are specific for DGAT or common for acyltransferases involved in the TG synthetic pathway. Niacin had no effect on MGAT activity, suggesting the specificity of niacin's effect for DGAT but not for MGAT and/or other acyltransferases within the TG synthetic pathway.

We performed additional studies examining the enzyme kinetic parameters in the absence and presence of niacin to determine apparent K_m, reaction velocity, and competitive/noncompetitive type of enzyme inhibition. We observed apparent K_m values of 8.3 and 100 μM for oleoyl-CoA and *sn*-1,2-dioleoylglycerol, respectively, for DGAT in HepG2 cell microsomes. Using palmitoyl-CoA as a substrate, previous studies indicated K_m values of 25 and 6 μM in rat liver microsomes and *Mycobacterium smegmatis*, respectively (29, 30). The data from LB plots indicate that niacin decreased the apparent V_{max} compared with controls, whereas the apparent K_m remained constant (Figs. 2A,B, 3A,B). The increase in concentration of oleoyl-CoA or DAG did not overcome niacin's inhibitory effect, suggesting that the DGAT inhibition by niacin is not through the competitive type of enzyme inhibition. Furthermore, the pattern of LB plots for control and niacin suggest that the inhibition of DGAT activity by niacin follows a noncompetitive type of enzyme inhibition. We suggest that niacin binds to DGAT at a site other than the enzyme active site and brings about a decrease in enzyme activity, which is a characteristic feature of noncompetitive enzyme inhibition. Because DGAT kinetic studies were done in the presence of 8 mM $MgCl_2$, the kinetic data obtained in our studies represent both DGAT1 and DGAT2. Coleman and associates (29) have shown that 2-bromopalmitoyl-CoA inhibited DGAT activity by noncompetitive enzyme inhibi-

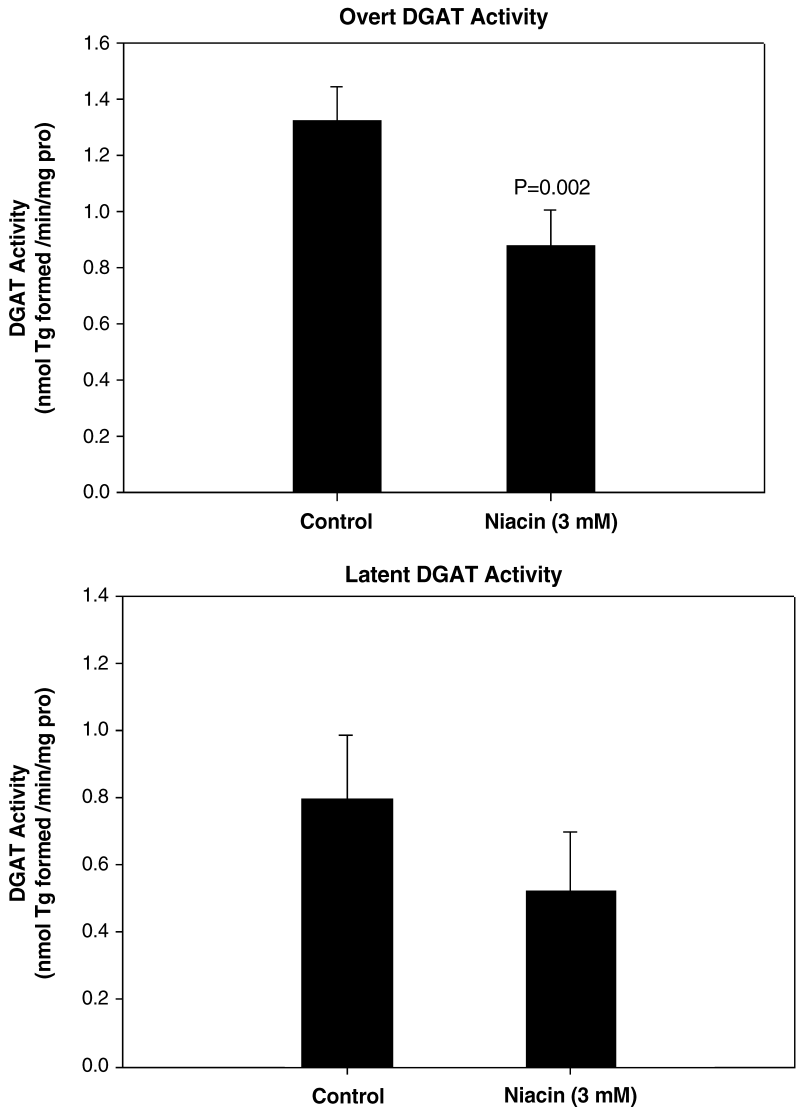

Fig. 5. Effect of niacin on overt and latent DGAT activity. DGAT activity was measured in HepG2 cell microsomes before and after permeabilization of the membrane using alamethicin. The data shown are means ± SEM of three determinations of a representative experiment. Three such experiments were performed, and similar results were obtained. The *P* value indicated refers to overt DGAT activity compared with control.

tion. Previously, it was shown that rat liver microsomal DGAT required 20 μM palmitoyl-CoA to attain V_{max}, and greater than 20 μM palmitoyl-CoA inhibited microsomal DGAT activity (31). However, such DGAT inhibition by high concentrations of palmitoyl-CoA was not observed in partially purified rat liver DGAT (31). We have also noted similar inhibition of HepG2 cell microsomal DGAT activity by high concentrations of oleoyl-CoA (>10 μM) and *sn*-1,2-dioleoylglycerol (>125 μM). Further work is needed to understand whether such substrate-mediated DGAT inhibition has a role in limiting TG production by excessive free fatty acid mobilization so as not to overwhelm the secretory capacity of the liver.

Two distinct DGAT1 and DGAT2 genes from different gene families regulate TG synthesis in mammalian cells (12, 14, 15). DGAT1 is highly expressed in the small intestine, and DGAT1 accounts for the majority (~90%) of intestinal DGAT activity (14, 32). Intestinal triacylglycerol absorption occurs by a series of steps in which dietary TGs are first hydrolyzed in the intestinal lumen and then resynthesized within enterocytes by DGAT-catalyzed reaction before incorporating into nascent chylomicrons and entering the lymphatic system. Studies indicated that DGAT1-deficient mice had reduced postabsorptive chylomicronemia and accumulated neutral lipid droplets in the cytoplasm of enterocytes when chronically fed a high-fat diet (32). These findings suggest a reduced rate of TG absorption in the DGAT1 deficiency state. Additional analysis of intestines from DGAT1-deficient mice revealed activity for DGAT2, and the DGAT2 appears to compensate for the absence of DGAT1 for TG synthesis and absorption (32). DGAT1-deficient mice have reduced body triacyl-

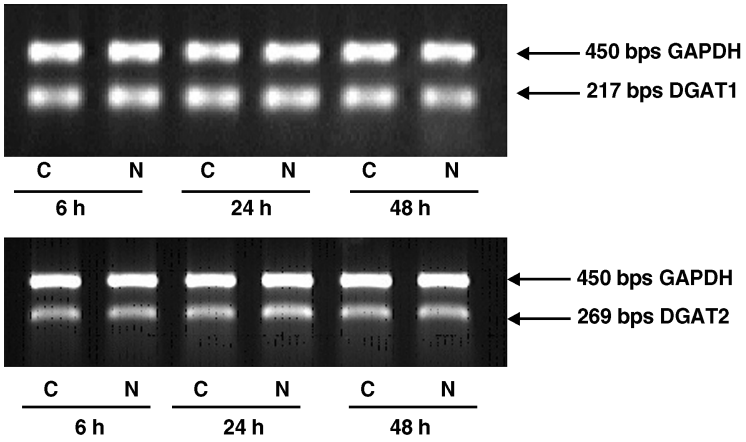

Fig. 6. Effect of niacin on the expression of DGAT mRNA. HepG2 cells were incubated in the absence and presence of 3 mM niacin for 6, 24, and 48 h. Total RNA was isolated as described in Materials and Methods. The isolated RNA was subjected to semiquantitative RT-PCR analysis. After 24, 28, 32, and 36 cycles, samples were collected and run on a 1.2% agarose gel. The blot shown is from samples obtained at 28 cycles of amplification of DGAT1 (linear phase) and 32 cycles of amplification of DGAT2 (linear phase). C, control; N, 3 mM niacin. Lanes 1 and 2 correspond to control and 3 mM niacin treatment for 6 h. Lanes 3 and 4 correspond to control and 3 mM niacin treatment for 24 h. Lanes 5 and 6 correspond to control and 3 mM niacin treatment for 48 h. The data shown are from a representative experiment. Similar results indicating no effect were obtained in three separate experiments.

glycerol content and are resistant to obesity through a mechanism involving increased energy expenditure, suggesting that selective inhibition of DGAT1-mediated TG synthesis may be useful for treating obesity (33). Contrary to DGAT1, the highest levels of DGAT2 expression in human tissues were in liver and white adipose tissue (14). Lower levels of DGAT2 expression were found in mammary gland, testis, and peripheral blood leukocytes. Based on the expression profile and reduced TG absorption rate in DGAT1-deficient mice, DGAT1 has been suggested to be mainly involved in intestinal TG absorption. It has been suggested that the abundant expression of DGAT2 in liver may play an important role in hepatic TG synthesis destined for VLDL assembly and secretion (34). Additional direct studies using DGAT2 overexpression and/or DGAT2-deficient mice are needed to determine the role of DGAT2 in VLDL assembly and secretion. Our studies on DGAT1 and DGAT2 demonstrate that niacin selectively inhibited DGAT2 activity (Fig. 4). It is interesting that niacin did not inhibit DGAT1 activity. Because HepG2 cells exhibited both DGAT1 and DGAT2, our data appear to have selectivity toward DGAT2. However, additional future studies are warranted to examine niacin's inhibitory effect in DGAT1- and DGAT2-overexpressed baculovirus-infected insect cells. Based on the expression profile and proposed role, we suggest that the selective and powerful inhibition of DGAT2 by niacin may have an important role in the niacin-mediated inhibition of hepatic TG synthesis resulting in increased intracellular apoB degradation (7) and decreased VLDL secretion seen in patients treated with niacin (11).

It is suggested that overt and latent DGAT activities are involved in the synthesis of TG for cytosolic droplet and VLDL lipidation, respectively (16, 17). Therefore, the effect of niacin on latent and overt DGAT activities was studied. Assessment of the overt and latent DGAT activities indicated that niacin inhibited both overt and latent DGAT activity by ~30%. Although the overt and latent DGAT activities are suggested to be involved in the TG synthesis associated with the cytoplasmic lipid droplets and luminal VLDL assembly, respectively, there is no clear experimental evidence to show the relative contribution of these two DGAT activities in the intracellular lipidation of apoB, intracellular apoB degradation, and the assembly and secretion of VLDL particles. Furthermore, it is important to note that the roles of the two DGAT activities are likely to be complementary, as cytosolic droplet TG turns over rapidly at a rate 20-fold higher than the rate of TG secretion in cultured rat hepatocytes (35). It has also been shown that the hydrolysis of cytosolic TG provides substrates not only for oxidation but also for the synthesis of secreted TGs in the form of both acyl-CoA and partial glycerides (18, 35). Thus, the overall inhibition of overt and latent DGAT activities by niacin may contribute to TG synthesis within the cytosolic and luminal side of the ER membrane that may regulate niacin-mediated apoB lipidation and VLDL secretion.

Additional studies on mRNA transcription indicated that niacin had no effect on the mRNA expression of DGAT1 and DGAT2. Parallel with these mRNA levels, microsomes isolated from niacin-treated HepG2 cells (niacin treatment for 48 h) had similar DGAT activity as microsomes from control HepG2 cells. These data suggest that niacin, through noncompetitive enzyme inhibition but not by altering DGAT transcriptional message, de-

TABLE 1. Quantitative analysis of the effect of niacin on the expression of DGAT1 and DGAT2 mRNA in HepG2 cells

Incubation Time	Control DGAT1 and DGAT2	3 mM Niacin		*P*
		DGAT1	DGAT2	
h	%	*% change*		
6	100	109.46 ± 6.17	98.85 ± 5.87	NS
24	100	94.49 ± 2.27	103.84 ± 8.48	NS
48	100	104.73 ± 3.67	103.49 ± 1.32	NS

DGAT, diacylglycerol acyltransferase. Treatment of cells, RNA isolation, and RT-PCR procedures were as described for Fig. 6 and in Materials and Methods. The band intensity of DGAT and the internal standard GAPDH was measured using EagleSight software. DGAT1 and DGAT2 band intensity units were normalized with the GAPDH message. The data shown represent percentage change over control of the ratio of band intensity of samples obtained at 28 cycles of amplification of DGAT1 and 32 cycles of amplification of DGAT2 (linear amplification phase) cDNA. Data shown are means ± SD of three separate experiments.

creased DGAT activity. This inhibitory effect of niacin on microsomal DGAT activity may be of considerable mechanistic importance, as the rate of TG synthesis in the proximity of lipoprotein assembly sites regulates the lipidation of apoB and the subsequent assembly and secretion of VLDL/LDL particles (36–38). Niacin-mediated in vitro inhibition of DGAT activity in HepG2 cell microsomes may, at least in part, explain the observed reduction of plasma levels of TGs by 30–40% in niacin-treated patients.

The inhibitory effects of niacin on fatty acid synthesis and inhibition of peripheral lipolysis (e.g., adipose tissue) and decreased fatty acid mobilization may be additional mechanisms of action to explain TG reduction by niacin (8, 39–41). In the absence of other regulatory events, niacin-mediated inhibition in adipocyte fatty acid mobilization over prolonged steady-state conditions would result in excessive synthesis of TGs and obesity. However, this mechanism is unlikely because niacin is not known to affect body weight clinically. Based on our observations in hepatocytes, it is likely that niacin may also inhibit DGAT2 activity in adipocytes. This may provide a regulatory mechanism to limit excessive adipocyte TG synthesis and related obesity events in the face of the effect of niacin on adipocyte fatty acid mobilization.

Although the biological actions of niacin are not clearly known to be mediated by receptor-mediated events, recent reports indicate that the orphan G-protein-coupled receptor mouse PUMA-G and human HM74 act as cellular receptors for niacin (42, 43). PUMA-G and its human ortholog HM74 are highly expressed in white and brown adipose tissue and also are detected in various other tissues, including lung, adrenal gland, and spleen (42). However, PUMA-G and HM74 were not detected in liver tissue (42). Using PUMA-G-deficient mice, Tunaru et al. (42) demonstrated that PUMA-G mediates the niacin-induced inhibition of free fatty acid release from isolated adipocytes. Because of the lack of PUMA-G and/or HM74 in liver tissue, it is very unlikely that PUMA-G/HM74 mediates the effect of niacin on DGAT activity and TG synthesis in hepatocytes.

In summary, we demonstrate for the first time that niacin noncompetitively and directly inhibits the activity of DGAT2 but not DGAT1. This inhibition of DGAT2 activity in liver may result in a decreased rate of TG synthesis and its availability for intracellular apoB lipidation and translocation across the ER membrane, resulting in observed effects of increased apoB degradation (7). Thus, these results suggest DGAT2 as a major site of action of niacin to inhibit TG synthesis, resulting in decreased secretion of apoB-containing atherogenic lipoproteins. Niacin may represent a new therapeutic class of drugs that regulate atherogenic apoB-containing lipoprotein (e.g., VLDL and LDL) secretion by specifically targeting DGAT2.

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