Effects of γ-Tocotrienol on ApoB Synthesis, Degradation, and Secretion in HepG2 Cells

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Abstract—γ-Tocotrienol (γ-T3), a naturally occurring analog of tocopherol (vitamin E), has been shown to have a hypocholesterolemic effect in animals and humans. Unlike tocopherol, it has also been shown to reduce plasma apoB levels in hypercholesterolemic subjects. The aim of this study was to define the mechanism of action of γ-T3 on hepatic modulation of apoB production using cultured HepG2 cells as the model system. HepG2 cells preincubated with γ-T3 were initially shown to inhibit the rate of incorporation of [14C]acetate into cholesterol in a concentration- and time-dependent manner, with a maximum 86±3% inhibition at 50 μmol/L observed within 6 hours. γ-T3, on the other hand, had no significant effect on the uptake of [14C]glycerol into pools of cellular triacylglycerol and phospholipid relative to untreated control. The rate of apoB synthesis and secretion was then studied by an [35S]methionine pulse-labeling experiment and quantified by immunoprecipitating apoB on chasing up to 3 hours. An average reduction of 24±3% in labeled apoB in the media was apparent with γ-T3 despite a 60±2% increase in apoB synthesis. Fractionation of secreted apoB revealed a relatively denser lipoprotein particle, suggesting a less stable particle. Using a digitonin-permeabilized HepG2 cell system, the effects of γ-T3 on apoB translocation and degradation in the endoplasmic reticulum were further investigated. The generation of a specific N-terminal 70-kDa proteolytic fragment proved to be a sensitive measure of the rate of apoB translocation and degradation. The abundance of this fragment increased significantly in γ-T3-treated cells relative to untreated control cells (50±21%) after 2 hours of chase. In addition, the presence of γ-T3 resulted in an average decrease of 64±8% in intact apoB. Taken together, the data suggest that γ-T3 stimulates apoB degradation possibly as the result of decreased apoB translocation into the endoplasmic reticulum lumen. It is speculated that the lack of cholesterol availability reduces the number of secreted apoB-containing lipoprotein particles by limiting translocation of apoB into the endoplasmic reticulum lumen. (Arterioscler Thromb Vasc Biol. 1999;19:704-712.)

Key Words: apoB ■ tocotrienol ■ tocopherol ■ HMG-CoA reductase inhibitor ■ degradation

Apolipoprotein B-100 (apoB), the major structural component of VLDL and LDL, is an important risk factor for coronary artery disease (CAD). Many patients with CAD have been found to have abnormally high production rate of VLDL- and LDL-apoB. Therefore, overproduction of apoB-Lp by the human liver is an important contributing factor in the development of CAD.

Numerous studies have shown that hepatic production apoB-Lp can be regulated by diet, hormones, and drugs. The specific mechanism involved in the regulation of hepatic apoB-Lp secretion and faulty overproduction appears to be at the cotranslational and postranslational levels, as nascent apoB molecules are either secreted or degraded intracellularly. Evidence suggests that translocation efficiency across the endoplasmic reticulum (ER) is a key determining factor of apoB-Lp secretion. It is well documented that this point of regulation determines the amount of nascent apoB that enters the secretory pathway and how much is degraded. ApoB, which is efficiently translocated across the ER membrane, assembles into a lipoprotein particle and is secreted through the secretory pathway. On the other hand, any apoB that is not translocated across the ER membrane fails to assemble into a lipoprotein particle and is diverted into a degradative pathway. An ER-localized proteolytic system has been found to be responsible for degrading apoB, as both an ER luminal cysteine protease and a cytosolic ubiquitin-proteosome pathway have been implicated in the intracellular degradation of apoB. It is generally accepted that incomplete translocation results in a significant amount of apoB exposed to the cytosolic side of the ER membrane with the N-terminal end of apoB partially translocated into the ER lumen. This membrane-bound apoB is suggested to come into contact with components of the ubiquitin-proteosome pathway, releasing an N-terminal 70-kDa apoB fragment into the ER lumen. This N-terminal fragment is apparently produced from partially translocated apoB and has

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been used as an indirect indicator of inefficient translocation in a number of studies.\textsuperscript{15,16}

It is generally believed that the supply or availability of lipid components in the ER plays an important role in regulating the assembly and secretion of apoB-Lp. Both dietary and hormonal factors known to influence lipid availability have been shown to modulate the rate of apoB translocation, degradation, and secretion in cultured hepatocytes (reviewed in Reference 17). Oleic acid, for example, was shown to stimulate apoB secretion through an inhibition of protein degradation.\textsuperscript{18} The protection by this fatty acid appeared to be the result of increased triacylglycerol (TAG) synthesis. Under these conditions, the rate of apoB translocation is thought to accelerate, and apoB is sorted for assembly into a lipoprotein particle destined for secretion.\textsuperscript{19,20}

On the other hand, insulin was shown to enhance apoB degradation, possibly by preventing lipid interaction with apoB, rendering the partially translocated apoB susceptible to specific protease(s).\textsuperscript{21,22} Thus, it is widely accepted that lipid availability is obligatory for the assembly of apoB-Lp. Without lipids, apoB remains unprotected and is degraded. Despite the many factors known to regulate apoB-Lp secretion, there are still other factors that remain unknown.

One such factor is tocotrienol, a naturally occurring analog of tocopherol (vitamin E) found mainly in cereal grains and palm oil. Tocotrienols differ from tocopherol by possessing double bonds in the phytol tail. They are further divided into isomers (α, β, γ, and δ) on the basis of the degree and placement of the methyl substitution around the chromanol ring.\textsuperscript{23} Unlike tocopherol, tocotrienol has been shown to have an intrinsic hypcholesterolemic activity in animals and man.\textsuperscript{24,25} The favorable cholesterol-lowering profile of tocotrienols was attributed mainly to their downregulation of the rate-limiting enzyme of the cholesterol biosynthetic pathway, HMG-CoA reductase (HMGR).\textsuperscript{26} In vitro models later identified γ-tocotrienol (γ-T3) and δ-T3 to be more potent than α-T3 in suppressing cholesterol biosynthesis.\textsuperscript{23} When assayed in hypercholesterolemic human subjects, Palmvitee (Palm Oil Research Institute of Malaysia), a tocotrienol-rich fraction (TRF) of palm oil, and purified γ-T3 were shown to mediate decreases in plasma total cholesterol, LDL-cholesterol, and apoB levels.\textsuperscript{27} Similar results were shown with a novel TRF from rice bran oil with also a reduction in the thrombogenic Lp(a) plasma levels.\textsuperscript{28}

The beneficial effects of γ-T3 on plasma LDL-cholesterol and apoB levels deserve further investigation. Indeed, γ-T3 was shown to upregulate LDL receptor in mammalian cells and may be implicated in part for the reduction of apoB-Lp in vivo.\textsuperscript{26} However, the production rate of apoB-Lp may also be affected by γ-T3. Numerous studies have suggested that HMGR inhibitors can also reduce apoB secretion by limiting the availability of lipids (reviewed in Reference 29). The purpose of our study was to investigate the effects of γ-T3 on the secretion of apoB using the human hepatoma cell line, HepG2, as the model system. This cell line has been used extensively to study hepatic apoB-Lp secretion.\textsuperscript{30,31}

In our experiments, we used both intact and semipermeabilized HepG2 cells\textsuperscript{32} to study cellular processes such as synthesis, ER translocation, degradation, and secretion of apoB.

In this article, we have shown that γ-T3 inhibited the secretion of apoB despite a stimulation in apoB synthesis.

The discrepancy is accounted for by a net increase in the intracellular degradation of apoB in the ER, which prevented apoB from being secreted. These findings suggest that the decreased plasma LDL-cholesterol and apoB levels mediated by γ-T3 in vivo may be partly the result of decreased apoB secretion.

**Methods**

**Materials**

γ-T3 was provided by Palm Oil Research Institute of Malaysia, Kuala Lumpur. Stock solutions of γ-T3 were freshly prepared in 100% DMSO and preserved at −25°C for no longer than 3 weeks. Immediately before use, the stock solution was diluted in culture medium to give a final DMSO concentration of 0.1% (vol/vol). Experiments were performed in subdued light. The preparation was found to be pure by HPLC (data not shown).

HepG2 cells (HB 8065) were obtained from American Type Culture Collection. Cell culture media, FBS (certified grade), antibiotic-antimycotic mixture, and immunoprecipitin (10% wt/vol) were from Life Technologies Inc. Culture dishes and flasks were obtained from Corning Costar Corp. Digitinolin (50% purity), lipoprotein-deficient FBS (LDS), α- and γ-tocopherol, rabbit anti-goat IgG, goat IgG, protease inhibitors, and other common laboratory reagents were from Sigma Chemical Co. Electrophoresis reagents were from Bio-Rad Laboratories. [1,3-\textsuperscript{14}C]glycerol (1175 Ci/mmol), [1-\textsuperscript{14}C]acetic acid, sodium salt (40 to 60 mCi/mmol), [1,3,\textsuperscript{14}C]glycerol (>40 mCi/mmol), ENHANCE and Reflection autoradiography films were purchased from NEN Life Science Products. Monospecific goat anti-human apoB antiserum and affinity-purified apoB polyclonal antibody were obtained from Medix-Biotech Inc. Plastic-backed thin-layer chromatography (TLC) plates (silica gel 60) were from Alttech Associates Inc.

**Growth of HepG2 Cells**

Monolayer cell cultures were maintained in RPMI-1640 medium with 10% FBS at 37°C with 5% CO\textsubscript{2} and 95% air and subcultured in 35- or 60-mm-diameter dishes to about 80% confluence. Once confluence was reached, cells were first supplemented with 7% LDS in RPMI-1640 (LDS-RPMI) for 16 hours to induce cholesterol-ogenesys.\textsuperscript{29} After induction, cell cultures were treated with γ-T3 in LDS-RPMI. Untreated control cells received 0.1% (vol/vol) DMSO without γ-T3.

**Analysis of Cellular and Secreted Lipids**

To measure the rates of cholesterol and cholesterol ester (CE) synthesis and secretion, treated and untreated cells were labeled with 5 μCi/mL \textsuperscript{14}C]acetate for 2 or 6 hours. TAG and phospholipids synthesis and secretion, on the other hand, were labeled with \textsuperscript{14}C]glycerol for 2 or 6 hours. After labeling, the medium was collected and the cells were washed twice with cold PBS. Cellular and media lipids were then extracted with hexane-isopropanol (3:2, vol/vol) as described by Goldstein et al.\textsuperscript{33} The organic solvent was evaporated, resuspended in hexane, and spotted on a TLC plate. Neutral and polar lipids were separated using a 2-solvent system. Plates were first developed in chloroform-methanol-2% acetic acid-water (70:30:12:6:2) and then developed in ether-diethylether-glacial acetic acid (90:10:1). The TLC plates were dried, mixed in scintillation cocktail, and counted on a Packard Tri-Carb model 1500 liquid scintillation counter. After lipid extraction, cell proteins were digested in 1 mL of 0.1 mol/L NaOH and measured as described below.

**Pulse-Chase Experiments**

Treated and untreated HepG2 cells were preincubated in methionine-free RPMI for 15 minutes and pulsed with an [\textsuperscript{35}S]methionine labeling medium (100 μCi/mL of [\textsuperscript{35}S]methionine in methionine-free RPMI 7% LDS, with and without γ-T3) for 15 minutes. After the short pulse, the cells were washed with Hanks’ balanced salt-solution (HBSS) and chased in 7% LDS-RPMI-1640 supplemented with...
5 mmol/L methionine, with and without γ-T3. At various chase times, duplicate 35-mm dishes were harvested and cells were lysed in solubilization buffer (PBS containing 1% NP40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, 100 kallikrein-inactivating units/mL aprotinin, 0.1 mmol/L leupeptin, 5 μmol/L ALLN, [N-acetyl-leucyl-leucyl-norleucinal]). The lysates were centrifuged for 5 minutes in a microfuge (7500g), and the supernatants were collected for immunoprecipitation. Media collected at each time point were spun as above to remove any cell debris and mixed with a protease inhibitor cocktail (2 mmol/L PMSF, 100 kallikrein-inactivating units/mL aprotinin, 0.1 mmol/L leupeptin, 5 μmol/L ALLN, final concentration) before immunoprecipitation.

**Immunoprecipitation.**

Solubilized cell extracts, media, and fractions collected from sucrose gradients were diluted with PBS and 5% C buffer (0.25 mol/L Tris, pH 7.4, 0.75 mol/L NaCl, 0.025 mol/L EDTA, 5% Triton X-100) and were immunoprecipitated by the addition of an excess amount of goat anti-human apoB antiserum. Immunoprecipitation was allowed to proceed overnight at 4°C. After incubation, excess amount of rabbit anti-goat IgG was added, and the mixture was further incubated for 1 hour at 4°C. Immunoprecipitin (10% wt/vol) was added, and the incubation continued for 1 hour on a shaker at room temperature. The immunoprecipitin-antibody complex was then separated by centrifugation and washed extensively with an immunoprecipitation wash buffer (0.14 mol/L NaCl, 0.02 mol/L sodium dihydrogen phosphate, pH 7.5). Finally, the immunoprecipitates were prepared for SDS-PAGE by suspending and boiling in 60 μL of electrophoresis sample buffer.

In the degradation assay, a preimmunoprecipitation step was used to eliminate nonspecific binding. Samples were preclarified once with goat serum/immunoprecipitin before immunoprecipitation with an affinity-purified apoB polyclonal antibody.

**SDS-PAGE and Fluorography.**

SDS-PAGE was performed essentially as described. Gels were composed of 3% (wt/vol) stacking and 6% or 7% (wt/vol) resolving gels. Electrophoresis was at 60 V for 16 hours. The gels were fixed, stained, and fluorographed by incubating in ENHANCE. The gels were then exposed to an autoradiography film and incubated at −80°C for 1 to 3 days. Radiolabeled proteins visualized on the fluorographs were quantified by cutting the corresponding band from the dried gel, mixing in a scintillation cocktail, and counting on a Packard Tri-Carb model 1500 liquid scintillation counter.

**TCA Precipitation.**

The incorporation of [35S]methionine into total cell protein was determined by trichloroacetic acid (TCA) precipitation. An aliquot of the sample was spotted on GF/C filters, and the filters were washed twice in 10% (wt/vol) TCA at 60°C for 5 minutes and in absolute ethanol for 2 minutes. Radioactivity was measured on a Packard Tri-Carb model 1500 liquid scintillation counter after placing the filters in 5 mL of scintillation cocktail.

**Other Methods.**

Cell protein content was measured according to Bradford using BSA as the standard. The activity of lactate dehydrogenase was measured on a Beckman Synchron CX4 automated analyzer using a modification of the method of Gay et al.

**Statistical Analysis.**

Data were normalized to the amount of cellular protein. Statistical differences were analyzed by using paired Student’s t test with the level of significance set at P<0.05.

**Results.**

**γ-T3, but not Tocopherol, Inhibits Cholesterol Synthesis in a Dose- and Time-Dependent Manner.**

Initial studies were performed to determine an optimal concentration of γ-T3 that would inhibit de novo cholesterol synthesis without altering cell viability. As shown in Figure 1, γ-T3 added in various concentrations to the culture medium for 6 hours decreased the rate of incorporation of [3H]acetate into cellular cholesterol in a dose-dependent manner. Percent inhibition was 42±2% at 2 μmol/L, 54±4% at 10 μmol/L, 71±3% at 25 μmol/L, 86±3% at 50 μmol/L, and 93±2% at 75 μmol/L. A preliminary experiment showed γ-T3 was significantly more active in suppressing cholesterol synthesis when cells were incubated with LDS than without LDS (data not shown). For this reason, we chose to pretreat our cells with LDS to maximize the effects of γ-T3 on cholesterol synthesis. From the results obtained, the optimal inhibition was observed at 50 μmol/L. At this concentration, there was no significant release of lactate dehydrogenase into the media (data not shown). Furthermore, TCA-precipitable radioactivity from cells and medium incubated with 50 μmol/L γ-T3 increased slightly (see below), indicating that the vitamin did not have any deleterious effect on cell metabolism. The decrease in total cholesterol was accompanied by a decrease in both free cholesterol and esterified cholesterol, which reached 85±2% and 80±3% of control, respectively. In all further experiments, 50 μmol/L γ-T3 was used as the optimal concentration.

Time-course studies of HepG2 cells preincubated with 50 μmol/L γ-T3 were also performed similarly as described above. In these studies, cells were incubated and labeled with a [14C]acetate labeling medium with and without γ-T3 for a total of 2 hours, 6 hours, and 18 hours. The inhibitory effect of γ-T3 on newly synthesized cholesterol synthesis was
found to be time-dependent with an optimal inhibition observed within 6 hours (data not shown). In all further experiments, a 6-hour treatment was used.

In further support of the specific effect of γ-T3 on cholesterol synthesis, α-tocopherol and γ-tocopherol added at 50 μmol/L, for 6 hours were studied for its effect on de novo cholesterol synthesis. As expected, the incorporation of [14C]acetate into cholesterol remained essentially the same as the untreated control (P>0.05, n = 4) (Table 1). Similarly, combination of γ-T3 and α-tocopherol were also examined to test for possible additive effects. When assayed together, α-tocopherol did not increase the activity of γ-T3. To the contrary, there was a slight decrease in cholesterol synthesis, but it did not reach statistical significance when compared with the effects of γ-T3 treatment alone.

### Table 1. Effects of Tocotrienol, Tocopherol, and Binary Mixtures on Cholesterol Biosynthesis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total Cholesterol</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39613 ± 4202</td>
<td></td>
</tr>
<tr>
<td>γ-T3</td>
<td>9754 ± 943</td>
<td>-75*</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>38821 ± 420</td>
<td>-2</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>40246 ± 250</td>
<td>1.6</td>
</tr>
<tr>
<td>α-Tocopherol/γ-T3</td>
<td>14610 ± 2552</td>
<td>-63*</td>
</tr>
</tbody>
</table>

Hepatocytes were first incubated with 7% LDS-RPMI for 16 hours. Cells were then treated with α-tocopherol, γ-tocopherol, or α-tocopherol/γ-T3, all at a concentration of 50 μmol/L in LDS-RPMI for 4 hours. Untreated control cells received 0.1% (vol/vol) DMSO without vitamin E. Incubations were continued for 2 hours in the presence of 5 μCi/mL [14C]acetate. Total cellular cholesterol was determined by TLC. The results are expressed in disintegrations per minute per milligram cell protein. Each value is the mean ± SD of a typical experiment performed in duplicate. *Indicates P<0.05 vs control.

To evaluate the effects of γ-T3 on the synthesis of TAG and phospholipids, HepG2 cells were labeled with [14C]glycerol with and without 50 μmol/L γ-T3 for a total of 6 hours. As summarized in Figure 2, γ-T3 did not have an inhibitory effect on the accumulation of triglyceride and phospholipids in the cell (P>0.05, n=4).

**γ-T3 Also Inhibits Cholesterol Secretion**

Secretion of lipids into the culture media was further analyzed in cells treated with and without 50 μmol/L γ-T3. Results showed a similar response as that observed intracellularly. γ-T3 significantly inhibited the secretion of free cholesterol and CE by 77%±4% and 88%±3%, respectively (P<0.05, n=4). No appreciable effects on the secretion of TAG with γ-T3 were observed (P>0.05, n=4); however, phospholipid secretion was slightly elevated (15±3%; P<0.05, n=4). Together, the data suggest that the γ-T3-induced inhibition of cholesterol synthesis resulted in concomitant decrease in its secretion (summarized in Figure 2).

**γ-T3 Stimulates ApoB Synthesis, but Inhibits Its Secretion**

An initial metabolic pulse-chase labeling experiment was performed to assess the postsynthetic fate of nascent apoB grown in untreated control and γ-T3-treated cell cultures. Cells were pulsed with [35S]methionine for 10 minutes, and chased with excess cold methionine medium with and without γ-T3 for up to 180 minutes. Aliquots in the intracellular and extracellular fractions were collected at various times and analyzed. A preliminary experiment showed a 20 minutes’ delay in reaching peak incorporation of [35S]methionine into full-length apoB in both control and treated cells (data not shown). Figure 3A is the fluorograph with the corresponding line graph (Figure 3B) showing the amount of apoB synthesized and secreted by HepG2 cells in the presence and absence of γ-T3 of a typical pulse-chase experiment (repro-
A consistent increase in synthesis of both albumin (14% to 28%) and total protein (12% to 1%) secretion at the end of the chase. As shown in Figure 4B, the effect of γ-T3 on apoB synthesis and secretion was found to be specific.

**γ-T3-Treated HepG2 Cells Secrete Denser ApoB-Containing Lipoproteins**

The effect of γ-T3 on the density of secreted lipoprotein particles in HepG2 cells was also investigated. As depicted in Figure 5, cells incubated with γ-T3 secreted apoB-Lp with a density that was relatively denser than untreated control cells (fractions 2 to 5 represent HDL apoB-Lp, whereas fractions 6 to 12 represent the lower-density apoB-Lp). This appears to suggest that γ-T3 may interfere with the assembly and secretory processes of the apoB-Lp particle, resulting in a less stable particle. Although γ-T3 stimulated apoB secretion, the amount of apoB recovered in γ-T3-treated cells was slightly lower compared with untreated controls (data not shown).

**γ-T3 Stimulates ApoB Degradation by Decreasing ER Translocation**

The role of γ-T3 on apoB translocation and degradation was investigated using semipermeable HepG2 cells. This system has been shown to be specific for studying apoB degradation in the ER as generation of an N-terminal 70-kDa proteolytic fragment proved to be a sensitive measure of the rate of apoB degradation in the ER.

**TABLE 2. Changes in Intracellular ApoB During a 180-Minute Chase**

<table>
<thead>
<tr>
<th></th>
<th>Peak to 60 Minutes</th>
<th>Peak to 180 Minutes</th>
</tr>
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<tbody>
<tr>
<td>-γT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+γT3</td>
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The effect of γ-T3 on apoB degradation was investigated using semipermeable HepG2 cells. The results from 3 independent experiments are summarized in Figure 4B. Measurement of albumin was performed by immunoprecipitation as was apoB, and total protein was performed by TCA precipitation. A consistent increase in synthesis of both albumin (14% ± 4%) and total protein (12% ± 1%) secretion at the end of the chase. As shown in Figure 4B, the effect of γ-T3 on apoB synthesis and secretion was found to be specific.

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degradation.\(^{32,38}\) We further used this fragment as an indicator of incomplete translocation. In our assay, apoB translocation and degradation were monitored during a 120-minute chase in CSK buffer supplemented with and without 50 \(\mu\text{mol/L}\) of \(\gamma\)-T3. Figure 6 shows intact apoB (550 kDa) and its major 70-kDa apoB fragment immunoprecipitated at the beginning (0 minutes) and at the 120-minute chase time.

As shown in Figure 6, degradation of the 550-kDa intact apoB was observed in permeabilized cells with and without treatment. Interestingly, the appearance of the 70-kDa degradation fragment at the 120-minute chase was found to coincide with the loss of the intact apoB band. We then analyzed the data by measuring the level of intact apoB remaining at the 120-minute chase time and expressed it as a percentage of the amount at time 0. Percent intact apoB remaining was 67.4% in control cells and 32.6% in \(\gamma\)-T3-treated cells. When expressed as a percentage of treated cells over control, a 52% increase in apoB degradation was noted (average was found to be 64\(\pm\)8%, from 3 independent experiments performed in duplicate, versus control; \(P<0.05\)). Interestingly, the lower level of intact apoB remaining under \(\gamma\)-T3 treatment corresponded with an increased abundance of the 70-kDa fragment at the 120-minute chase. The fragment increased significantly in \(\gamma\)-T3-treated cells, relative to un-

Figure 6. Effect of \(\gamma\)-T3 on apoB translocation and degradation in the ER. Cells were treated as in Figure 3, pulsed with \([\text{35S}]\)methionine, chased, and permeabilized with digitonin in the presence or absence of \(\gamma\)-T3. Permeabilized cells were then incubated in CSK buffer for 120 minutes in the presence or absence of \(\gamma\)-T3. Cells were solubilized and analyzed by immunoprecipitation, SDS-PAGE, and fluorography. ApoB radioactivity was quantified by cutting the apoB band and counting it by scintillation. Intact apoB (550 kDa) and its 70-kDa degradation fragment are shown on the right. This figure represents a typical experiment performed 3 times in duplicate.

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Figure 5. Density distribution of secreted apoB-Lp in control and \(\gamma\)-T3-treated cells. Treated and untreated HepG2 cells were grown, pulsed, and chased for 3 hours as described in Figure 3. The extracellular fraction was collected at the end of the chase period (3 hours) and subjected to sucrose gradient ultracentrifugation. After centrifugation, gradient fractions were collected and immunoprecipitated with an anti-apoB antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography, and apoB radioactivity was quantified by cutting the apoB band and counting it by scintillation. This figure represents a typical experiment performed twice.
treated control cells, by nearly 2-fold. When taking the increase in intact apoB synthesis into account, an increase of 79% in the 70-kDa fragment was found (average was found to be 50±21%, from 3 independent experiments performed in duplicate, versus control; P<0.05). Taken together, the data suggest that γ-T3 stimulated apoB degradation in the ER by decreasing apoB translocation into the ER lumen.

Discussion
Factors that control the rate of apoB secretion by the liver have been of great importance in view of the role of apoB in atherogenesis. The purpose of this study was to investigate the effects of a dietary factor, γ-T3, on apoB secretion. The isolation of tocotrienols from palm oil and the making of synthetic derivatives have led to a new potential drug to treat hypercholesterolemia.23,39 This drug mimics other HMGR inhibitors; however, unlike HMGR inhibitors, γ-T3 is active against free radicals and is thought to inhibit LDL oxidation.39,40 Furthermore, in vitro studies have shown that tocotrienols exhibited greater antioxidant activity and antitumor activity than tocopherols.41,42 The beneficial effects of tocotrienols in cardiovascular disease and cancer are becoming more apparent.

In our study, we first examined the effects of γ-T3 on lipid synthesis and secretion in HepG2 cells. Our results confirmed that γ-T3, unlike α- and γ-tocopherol, is able to inhibit cholesterol synthesis in a dose- and time-dependent fashion. These results compare well with those of Parker et al.26 In their studies with γ-T3-treated HepG2 cells, they observed an inhibition in cholesterol synthesis of nearly 80% within 4 hours at a concentration of 30 μmol/L. In elucidating the mechanism of action, they found that the suppression was because of a posttranslational process involving an accelerated rate in the intracellular degradation of HMGR. These authors, however, did not investigate whether γ-T3 was exerting its effect primarily through CE or free, unesterified cholesterol. Our report showed that γ-T3 exerted an equal inhibitory effect on both esterified and free cholesterol synthesis. Furthermore, our results indicated a similar decrease in free cholesterol and CE accumulation in the media. On the other hand, the synthesis and secretion of TAG was not significantly altered by γ-T3. As for phospholipids, only secretion showed a slight increase with γ-T3, whereas synthesis remained essentially unchanged.

To further examine whether lipid availability is associated with apoB secretion, we investigated the effects of γ-T3 on apoB synthesis, degradation, and secretion with pulse-chase experiments using intact HepG2 cells. As shown, γ-T3 significantly reduced apoB secretion despite an excess amount of apoB in the cultured cell. It is postulated that the lack of lipid availability, primarily in esterified cholesterol, in γ-T3-treated cells, prevented lipid interaction with nascent apoB. Without lipids, it is thought that apoB molecules are unable to translocate across the ER membrane and are degraded intracellularly by the ubiquitin-proteosome pathway located on the cytosolic side of the ER membrane (reviewed in Reference 43). Interestingly, degradation was found to be rapid in γ-T3-treated cells relative to the untreated control cells, as nearly all of nascent apoB was degraded within the first hour of chase. In turn, apoB secretion was found to be inhibited by an average of 24±3% at the 3-hour chase time despite a 60±2% increase in apoB synthesis at the beginning of the chase (from 3 independent experiments performed in duplicate). Furthermore, γ-T3-treated HepG2 cells secreted denser apoB-Lp, suggesting that lipoprotein assembly may be hampered. Dense HDL-like apoB-Lp particles have been shown to be more unstable because they undergo a higher level of intracellular degradation than LDL-like particles.54 Together, the results implicate a beneficial role for γ-T3 in the regulation of apoB secretion and illustrate the importance of lipid availability in the secretion of apoB-Lp.

Simultaneously, we analyzed the metabolic fate of labeled albumin and total protein in both the intracellular and extracellular fractions. The synthesis and secretion of albumin and the total amount of newly synthesized protein were only slightly affected by γ-T3. Both the synthesis and secretion increased slightly indicating that the response with apoB was specific. The increase in protein synthesis, including apoB, is interesting and may reflect a global transcriptional effect. This is compatible with the mechanisms of action of tocopherols, which can influence RNA polymerase activity by interacting with tocopherol-binding protein,44 a response similar to vitamin A and the retinol-binding protein.

The site of apoB degradation has been of great interest. In HepG2 cells, the major site of apoB degradation is the ER.10,14 A cytosolic ubiquitin-proteosome pathway associated with the ER membrane has been largely implicated in the intracellular degradation of apoB in HepG2 cells.12 However, Adeli and coworkers13 further suggested that an ER luminal cysteine protease is also involved in a second degradative pathway crucial in controlling the rate of apoB secretion. To further elucidate the role of γ-T3 on apoB degradation, a specific ER degradation assay using semipermeabilized cells was used to examine its effect on apoB degradation. This assay has been described previously and has been used to study factors that modulate the rate of apoB translocation and degradation.45 It uses digitonin, which selectively permeabilizes the plasma membrane leaving the ER morphologically intact and functional. These semipermeabilized cells have the unique ability to allow cellular processes such as ER translocation, folding, and assembly of secretory proteins to occur as they would in the intact cell. Interestingly, the slower rate of apoB degradation in this system allows for the detection of a specific N-terminal 70-kDa proteolytic fragment.32 According to the current hypothesis of the apoB secretory pathway, generation of this fragment apparently results from inefficient apoB translocation across the ER membrane. Therefore, the appearance of the 70-kDa fragment was used in our study as a marker of apoB translocation and degradation. As shown in our report, the greater abundance of this fragment in γ-T3-treated cells at the 2-hour chase time suggested an increase in apoB degradation. Supporting this hypothesis, the appearance of the 70-kDa fragment correlated with less intact apoB remaining under γ-T3 treatment compared with the untreated control. Together, the present results are consistent with the pulse-chase and fractionation studies and provide further evidence that γ-T3 stimulates apoB degradation, possibly as the result of decreased translocation across the ER membrane.

Today, much controversy still exists as to which lipid is the primary constituent in the assembly of apoB-Lp. Both TAG and CE have been suggested as the lipids that primarily regulate apoB translocation and degradation; however, at-
tempts to distinguish which of these lipids play a more important role have yielded conflicting results. In the current study, we demonstrate that CE had the major regulatory role in the secretion of apoB. This agrees well with the work of Cianflone et al. and Musanti et al., who demonstrated that when an ACAT (acetyl CoA:cholesterol acyltransferase) inhibitor, a potent suppressor of cholesterol esterification, was added to HepG2 cells, the secretion of apoB-Lp dropped significantly. Additionally, pravastatin, a potent HMGCR inhibitor, was shown to reduce apoB secretion significantly. Additionally, pravastatin, a potent HMGR inhibitor, was shown to reduce apoB secretion by accelerating the intracellular degradation of apoB. This was coincident with a decrease in CE synthesis while free cholesterol and TAG synthesis remained unchanged. Similarly, simvastatin significantly reduced apoB secretion in HepG2 cells and paralleled changes in cholesterol synthesis. On the other hand, not all studies saw a requirement for CE synthesis in the regulation of apoB secretion. In a series of experiments in HepG2 cells, Wu et al. demonstrated that changes in cellular CE content did not affect apoB secretion, whereas changes in TAG synthesis were always associated with changes in apoB secretion. It was suggested that the availability of newly synthesized TAG was the most important factor to regulate apoB secretion. The data are in accord with previous studies in which various ACAT and HMGCR inhibitors had no effect on apoB secretion. Clearly, there are discrepancies. A recent study by Cartwright et al. argued that different pools of CE may be differentially modified by the various inhibitors used to alter CE, which may explain the discrepancies. Hence, it is plausible that γ-T3 affected CE availability early in the assembly process of apoB-Lp and that this particular pool of CE behaved as the primary constituent in regulating apoB secretion.

The failure of some hypercholesterolemic subjects to respond to γ-T3 have prompted investigators to evaluate the preparation and standardization of tocotrienol for human tests. Most human studies used a TRF of palm oil as their preparation. The composition of TRF is highly variable, and the tocopherol content can vary from 15% to 44%. It was observed that preparations with 20% or more tocopherol can attenuate the cholesterol-suppressive action of γ-T3 and may be responsible for conflicting reports on responses to TRF in human subjects. Our results (Table 1), as well as those of others, indicated that tocopherol did not significantly decrease the activity of γ-T3 in HepG2 cells. Therefore, it appears that the attenuation observed in vivo may be related to transport systems in the blood. Qureshi et al. hypothesized that there might be a preferential transport of α-tocopherol in serum lipoproteins.

In all, the data in this report indicate that γ-T3 influences apoB secretion by both cotranslational and posttranslational processes involving a decreased rate of apoB translocation and accelerated degradation of apoB. This activity correlated with a decrease in free and esterified cholesterol. Taken together, the information indicates an association between the suppression of hepatic cholesterol synthesis and apoB secretion, and the observed lowering of apoB and LDL-cholesterol levels in animal models.

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712 γ-Tocotrienol Stimulates ApoB Degradation

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