

Modulation of hepatic lipoprotein synthesis and secretion by taxifolin, a plant flavonoid¹

Andre Theriault,^{2,*} Qi Wang,^{*} Stephen C. Van Iderstine,[†] Biao Chen,[†] Adrian A. Franke,[§] and Khosrow Adeli[†]

Division of Medical Technology,^{*} University of Hawaii, Honolulu, HI 96822; Department of Laboratory Medicine,[†] Hospital for Sick Children, Toronto, Canada; and Cancer Research Center of Hawaii,[§] University of Hawaii, Honolulu, HI 96822

Abstract In the present study, the effects of taxifolin, a plant flavonoid, on lipid, apolipoprotein B (apoB), and apolipoprotein A-I (apoA-I) synthesis and secretion were determined in HepG2 cells. Pretreatment of cells with (\pm)-taxifolin led to an inhibition of cholesterol synthesis in a dose- and time-dependent manner, with an $86 \pm 3\%$ inhibition at $200 \mu\text{M}$ observed within 24 h. As to the mechanism underlying this inhibitory effect, taxifolin was shown to inhibit the activity of HMG-CoA reductase by $47 \pm 7\%$. In addition, cellular cholesterol esterification, and triacylglycerol and phospholipid syntheses, were also significantly suppressed in the presence of taxifolin. ApoA-I and apoB synthesis and secretion were then studied by pulse-chase experiments. ApoA-I secretion was found to increase by $36 \pm 10\%$. In contrast, an average reduction of $61 \pm 8\%$ in labeled apoB in the medium was apparent with taxifolin. This effect on secretion appeared not to be exerted at the transcriptional level. Rather, the effect on apoB secretion was found to be exerted in the early stages of apoB degradation and to be sensitive to dithiothreitol (DTT) and insensitive to *N*-acetyl-leucyl-leucyl-norleucinal, suggesting a proteolytic pathway involving a DTT-sensitive protease. Fractionation of secreted apoB revealed a slight shift in the distribution of secreted apoB-containing lipoproteins. Cholesteryl ester, rather than triacylglycerol, was shown to be the lipid that primarily regulated apoB secretion. **In summary, our data suggest that taxifolin decreases hepatic lipid synthesis with a concomitant decrease and increase in apoB and apoA-I secretion, respectively.**—Theriault, A., Q. Wang, S. C. Van Iderstine, B. Chen, A. A. Franke, and K. Adeli. **Modulation of hepatic lipoprotein synthesis and secretion by taxifolin, a plant flavonoid.** *J. Lipid Res.* 2000. 41: 1969–1979.

Supplementary key words apolipoprotein B • cholesterol • silymarin • silybin • HepG2

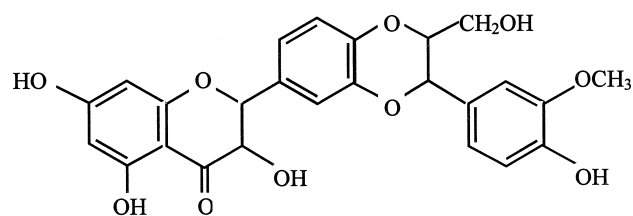
There has been a considerable amount of interest in the benefits of diets rich in flavonoid-containing foods, such as fruits, vegetables, wine, and tea, with respect to cardiovascular disease (CVD) and certain cancers (1). The protective effects of flavonoids against these chronic diseases have been attributed to their free radical-scavenging

property. In the case of CVD, flavonoids have been shown to reduce low density lipoprotein (LDL) oxidation, an important step in atherogenesis (2, 3). However, studies have given evidence about the potential role of these flavonoids in reducing blood lipid levels. Investigations by Choi, Yokozawa, and Oura (4) and Jahromi and Ray (5) showed that flavonoid components of *Prunus davidiana* stem extract and *Pterocarpus marsupium* heartwood extract reduced cholesterol levels in hyperlipidemic rats. In humans, the intake of soy protein rich in isoflavonoids was shown to significantly reduce serum cholesterol levels (6). In elucidating the molecular mechanism of flavonoid action on cholesterol homeostasis, at least two enzymes involved in cholesterol metabolism were shown to be involved. Work by Wilcox et al. (7) and Borradaile, Carroll, and Kurowska (8) provided evidence that the citrus flavonoids naringenin and hesperetin decreased cholesterol synthesis by inhibiting acyl-CoA:cholesterol acyltransferase (ACAT) activity in HepG2 cells. In contrast, Nassuato et al. (9) demonstrated in rat liver that silybin, the major flavolignan from the fruit of *Silybum marianum* (syn. *Carduus marianus* L.), reduced cholesterol synthesis by suppressing 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) activity, the rate-limiting enzyme in cholesterol synthesis. Bok et al. (10) showed in vivo that a combination of these

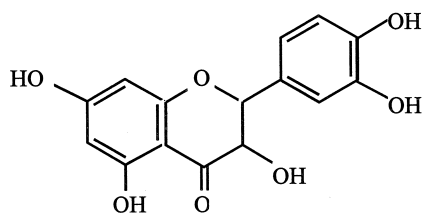
Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B-100; apoB-Lp, apoB-containing lipoprotein; ATCC, American Type Culture Collection; BSA, bovine serum albumin; CE, cholesteryl ester; CVD, cardiovascular disease; DTT, dithiothreitol; EBSS, Earle's balanced salt solution; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; HDL, high density lipoprotein; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDH, lactate dehydrogenase; LDL, low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SF-RPMI, serum-free RPMI; TAG, triacylglycerol; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

¹ Part of this work was presented at the American Heart Association 72nd Scientific Sessions, Atlanta, GA, in November 1999, and printed in abstract form in *Circulation*. 1999. 100: I-109.

² To whom correspondence should be addressed.



Silybin



Taxifolin

Fig. 1. Structures of silybin and taxifolin.

two lipogenic enzymes may account for the hypocholesterolemic effect of flavonoids. Despite these findings, it is unclear whether all naturally derived flavonoids (well over 4,000 of them) share these properties. Moreover, whether all flavonoid molecules function to the same degree in cholesterol synthesis remains to be addressed.

The effect of silybin on cholesterol metabolism was further studied in hypercholesterolemic subjects (9). Although biliary cholesterol concentrations were found to be reduced, the exact compound(s) responsible for this effect was disputed. Krecman et al. (11) studied in rats the hypocholesterolemic effects of both silymarin, a mixture of flavolignans extracted from *S. marianum*, and pure silybin, a single flavolignan. Interestingly, they found that silybin was not as effective as silymarin, suggesting that other constituent(s) of silymarin, in addition to silybin, may also have hypocholesterolemic effects. Subsequently, the minor constituent of silymarin, taxifolin, has attracted the attention of our laboratory, because taxifolin constitutes the flavonoid moiety in the flavolignan silybin (Fig. 1). Hence, the purpose of our study was to investigate the effects of taxifolin on lipids, apolipoprotein B-100 (apoB), and apolipoprotein A-I (apoA-I) synthesis and secretion, using a well-established human hepatoma cell-line, HepG2, as the model system.

MATERIALS AND METHODS

Materials

(±)-Taxifolin (>98% pure; purchased from Sigma, St. Louis, MO) was prepared in ethanol and preserved at -25°C for no longer than 4 weeks. Immediately before use, the stock solution was diluted in culture medium to give a final ethanol concentration of 0.1% (v/v). The concentration used was verified by molar absorptivity ($\epsilon = 19,953, 290 \text{ nm}$).

HepG2 cells (HB 8065) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cell culture media,

fetal bovine serum (certified grade), antibiotic-antimycotic mixture, and Trizol reagent were from Life Technologies (Grand Island, NY). Culture dishes and flasks were obtained from Corning Costar (Cambridge, MA). Electrophoresis reagents were from Bio-Rad (Hercules, CA). [^{35}S]protein labeling mix (1,175 Ci/mmol), [^{14}C]acetic acid (40–60 mCi/mmol), [$^{1,3-^{14}\text{C}}$]glycerol (>40 mCi/mmol), [$^{1-^{14}\text{C}}$]oleic acid (40–60 mCi/mmol), [$^{glutaryl-3-^{14}\text{C}}$]hydroxy-3-methylglutaryl coenzyme A (40–60 mCi/mmol), ENHANCETM, and ReflectionTM autoradiography films were purchased from NEN Life Science Research Products (Boston, MA). Monospecific goat anti-human apoB antiserum was obtained from Alexon-Trend (Ramsey, MN). Protein A-positive *Staphylococcus aureus* cells were from Roche Molecular Biochemicals (Indianapolis, IN). Plastic-backed thin-layer chromatography (TLC) plates (Silica Gel 60) were from Alltech Associates (Deerfield, IL). The CytoTox 96 nonradioactive cytotoxicity assay and the pGEM-7Zf vector were from Promega (Madison, WI). Other common laboratory reagents were from Sigma.

Cell culture

Monolayer HepG2 cell cultures were maintained in RPMI 1640 medium with 10% FBS at 37°C with 5% CO_2 and 95% air and subcultured in 35-mm-diameter dishes until about 80% confluency. Once confluency was reached, cells were treated with taxifolin in serum-free RPMI (SF-RPMI). Untreated control cells received 0.1% (v/v) ethanol without taxifolin.

Analysis of cellular and secreted lipids

To measure the rates of cholesterol and cholesteryl ester (CE) synthesis and secretion, treated and untreated cells were labeled with [^{14}C]acetate (5 $\mu\text{Ci/ml}$) for 6 h. Triacylglycerol (TAG) and phospholipids, on the other hand, were labeled with [^{14}C]glycerol (2.5 $\mu\text{Ci/ml}$) for 6 h. After labeling, the medium was collected and the cells were washed twice with cold phosphate-buffered saline. Cellular and medium lipids were then extracted with hexane-isopropanol 3:2 (v/v) as described by Goldstein, Basu, and Brown (12). The organic solvent was evaporated, and lipids were resuspended in hexane and spotted on a TLC plate. Neutral and polar lipids were separated using a two-solvent system. Plates were first developed in chloroform-methanol-acetic acid-formic acid-water 70:30:12:4:2 (v/v/v/v/v) and then developed in ether-diethyl ether-glacial acetic acid 90:10:1 (v/v/v). The TLC plates were dried and the lipids were visualized with I_2 vapor and zones corresponding to the lipid standard were cut, mixed in scintillation cocktail, and counted on a Packard (Downers Grove, IL) Tri-Carb model 1500 liquid scintillation counter. After lipid extraction, cell proteins were digested in 1 ml of 0.1 M NaOH and measured as described below.

Analysis of HMG-CoA reductase activity

HMGR activity was measured in permeabilized HepG2 cells according to Leonard and Chen (13). Briefly, treated and untreated cells, grown in 24-well culture plates, were permeabilized with digitonin as described by Theriault et al. (14). After permeabilization, cells were rinsed once with cytoskeletal buffer and immediately used for the HMGR assay. The permeabilized cells were first incubated in preincubation buffer [50 mM phosphate buffer (pH 7.4), 10 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA)] \pm taxifolin for 20 min at 37°C . The enzyme assay was initiated by adding labeling buffer [100 mM phosphate buffer (pH 7.4), 5 mM DTT, 20 mM glucose 6-phosphate, 2.5 mM NADP, glucose-6-phosphate dehydrogenase (3.3 units/ml), and [^{14}C]HMG-CoA (1 $\mu\text{Ci/ml}$, 150 μM)] \pm taxifolin for 30 min at 37°C . Finally, the reaction was terminated by the addition of 0.8 M HCl and further incubated for 30 min at 37°C to lactonize mevalonate. The [^{14}C]mevalono-

lactone formed from [^{14}C]HMG-CoA was isolated by TLC, using a protocol similar to that described above. Briefly, the supernatant was extracted with diethyl ether. After evaporation of the organic solvent, mevalonolactone was resuspended in chloroform–methanol 2:1 (v/v), and an aliquot was spotted on a TLC plate. Mevalonolactone was separated by development in benzene–acetone–acetic acid 400:600:1 (v/v/v) and visualized with I_2 vapor. The zone corresponding to the mevalonolactone standard was cut, mixed in a scintillation cocktail, and counted on a scintillation counter. Results (counts per minute) were converted into micromoles per minute per well and expressed relative to the values obtained from untreated control cells. An internal standard, [^3H]mevalonate, was added to the assay for estimating recovery.

Analysis of CE formation

Cellular cholesterol esterification was determined according to the method described essentially by Goldstein, Basu, and Brown (12). Briefly, each dish pretreated with or without taxifolin for 22 h received [^{14}C]oleic acid (0.5 $\mu\text{Ci}/\text{ml}$, 360 μM) complexed to fatty acid-free bovine serum albumin (BSA) and incubated for a further 2 h in the presence and absence of taxifolin. The molar ratio of oleic acid to BSA was 8:1. The cells were then washed three times with Earle's balanced salt solution (EBSS), the lipids were extracted *in situ*, and CE was separated by TLC as described above. Cell protein was determined as described below. An internal standard, [^3H]cholesterol oleate, was added to the assay to correct for procedural losses.

Metabolic pulse-chase labeling experiments

Treated and untreated HepG2 cells were preincubated in methionine/cysteine-free RPMI for 30 min and pulsed with an [^{35}S]protein labeling medium ([^{35}S]protein labeling mix at 100 $\mu\text{Ci}/\text{ml}$ in SF-RPMI \pm taxifolin) for 10 min. After the short pulse, the cells were washed with EBSS and chased in SF-RPMI 1640 supplemented with 5 mM methionine/cysteine \pm taxifolin. At various chase times, duplicate 35-mm dishes were harvested and cells were lysed in solubilization buffer as described previously (14). The lysates were centrifuged for 5 min in a microcentrifuge (7,500 g) and the supernatants were collected for immunoprecipitation. Media collected at each time point were spun as described above to remove any cell debris and mixed with a protease inhibitor cocktail [2 mM phenylmethylsulfonyl fluoride, aprotinin (100 kallikrein-inactivating units/ml), 0.1 mM leupeptin, and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN, 5 μM final concentration)] prior to immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography as described previously (14).

RNase protection assay for apoB and apoA-I mRNA abundance

ApoB and apoA-I mRNA levels were measured by an RNase protection/solution hybridization assay as described similarly by Azrolen and Breslow (15). This was performed by incubating the labeled probe ($3\text{--}9 \times 10^4$ cpm, 150 pg) with total cellular RNA (20 μg) in 40 μl of hybridization buffer [80% formamide, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 6.7), 0.4 M NaCl, 1 mM EDTA] overnight at 53°C (for apoB probe) or 63°C (for apoA-I probe). Unhybridized RNA was degraded by the addition of RNase A and RNase T1 in digestion buffer [0.3 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA] for 1 h at 34°C. Protected RNA was then precipitated with 20% trichloroacetic acid (TCA) and 100 μg of salmon sperm DNA for 15 min on ice. Each sample was filtered through glass fiber filters (GF/C) and washed with 10% TCA. Radioactivity was quantified by counting the filters in a liquid scintillation counter.

Human specific cDNA for apoB was kindly provided by Z. Yao (University of Ottawa, Canada), whereas apoA-I cDNA was purchased from the ATCC. Both amplified fragments were ligated to a pGEM-7zf vector, which served as the template to synthesize antisense RNA probes. A mouse β -actin construct was used to normalize total RNA amount. Unlabeled cRNA corresponding to the sense DNA strand was prepared for use as a hybridization standard.

Lipoprotein fractionation

Cells were treated and subjected to pulse-chase labeling as described above. The culture medium was collected after the 2-h chase, adjusted to 10% sucrose, and separated by sucrose gradient ultracentrifugation as previously described (14). Centrifugation was carried out at 35,000 rpm at 12°C for 65 h in an SW41 Ti rotor. Gradients were fractionated into 1-ml fractions, and the density and apoB were measured in each fraction. All solutions contained the protease inhibitor cocktail as above.

Other methods

Cell protein content was measured according to Bradford (16) (i.e., Bio-Rad), using BSA as the standard. The activity of lactate dehydrogenase (LDH) released into the medium was measured spectrophotometrically, using the CytoTox 96 non-radioactive cytotoxicity assay according to the manufacturer protocol (Promega).

Statistical analysis

Data were normalized to the amount of cellular protein. Statistical differences were analyzed with a paired *t*-test with the level of significance set at 0.05.

RESULTS

Taxifolin inhibits cholesterol synthesis in a dose- and time-dependent manner

Initial studies were performed to determine an optimal concentration of taxifolin that would inhibit *de novo* cholesterol synthesis without altering cell viability. As shown in **Fig. 2**, taxifolin added in various concentrations to the culture medium for 24 h decreased the rate of incorporation of [^{14}C]acetate into cellular cholesterol in a dose-dependent manner. Percent inhibition was $35 \pm 4\%$ at 50 μM , $54 \pm 5\%$ at 100 μM , and $86 \pm 3\%$ at 200 μM . At or below 200 μM , there was no significant release of LDH into the medium, indicating no cytotoxicity effect (data not shown). Furthermore, TCA-precipitable radioactivity from cells incubated with 200 μM taxifolin remained essentially unchanged versus untreated control indicating that the flavonoid did not alter cellular protein synthesis (data not shown). Consequently, this nontoxic pharmacologic dose was chosen in the following experiments. At 200 μM , the decrease in total cellular cholesterol was accompanied by a decrease in both free cholesterol and esterified cholesterol, which reached 85 ± 3 and $80 \pm 2\%$ of control, respectively.

Time course studies of HepG2 cells preincubated with 200 μM taxifolin were also performed. In these studies, cells were incubated and labeled with a [^{14}C]acetate labeling medium with and without taxifolin for a total of 6, 18, and 24 h. The inhibitory effect of taxifolin on newly synthe-

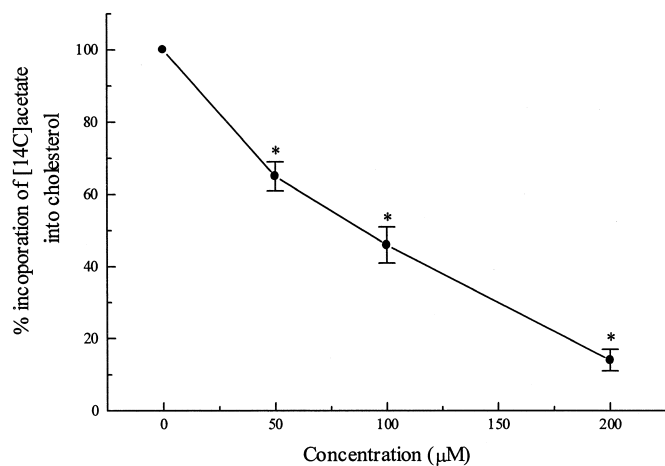


Fig. 2. Dose-dependent inhibition of incorporation of [^{14}C]acetate into cholesterol by taxifolin. Confluent, monolayer HepG2 cell cultures were supplemented with various concentrations of taxifolin in SF-RPMI for 18 h. Untreated control cells received 0.1% (v/v) ethanol without taxifolin. Incubations were continued for 6 h in the presence of [^{14}C]acetate (5 $\mu\text{Ci}/\text{ml}$). Total cellular cholesterol was then determined by TLC and scintillation counting. Data are expressed as percent incorporation (mean \pm SD) of [^{14}C]acetate into cholesterol of duplicate samples repeated in three separate experiments. * $P < 0.05$ versus control.

sized cholesterol synthesis was found to be time dependent, with an optimal inhibition observed within 24 h (data not shown). In all further experiments, a 24-h treatment was used.

Secretion of cholesterol into the culture medium was also analyzed simultaneously in cells treated with and without 200 μM taxifolin. Results showed a response similar to that observed intracellularly. Taxifolin significantly inhibited the secretion of free cholesterol and CE by 81 ± 1 and $80 \pm 5\%$, respectively ($P < 0.05$, $n = 3$). Together, the data suggest that the taxifolin-induced inhibition of cholesterol synthesis resulted in a concomitant decrease in its secretion (summarized in Fig. 4).

Taxifolin suppresses HMG-CoA reductase activity and CE formation

To determine the underlying mechanism of action of taxifolin on cholesterol synthesis, HMGR activity was measured by the rate of incorporation of [^{14}C]HMG-CoA into mevalonate in permeabilized cells. This method has been described previously and has been used to study factors that modulate HMGR activity (13, 17). It uses digitonin, which selectively permeabilized the plasma membrane, leaving the endoplasmic reticulum (ER) morphologically intact and functional. As shown in Fig. 3, taxifolin (200 μM , 24 h) reduced HMGR activity by $47 \pm 7\%$ ($P < 0.05$, $n = 3$). This result suggest that taxifolin produces its cholesterol-lowering effect through the inhibition of HMGR, the rate-limiting enzyme in cholesterol biosynthesis.

To examine further the effect of taxifolin on CE formation, cholesterol esterification was determined in situ by the rate of incorporation of [^{14}C]oleic acid into cellular CE. As shown in Fig. 3, taxifolin (200 μM , 24 h) reduced choles-

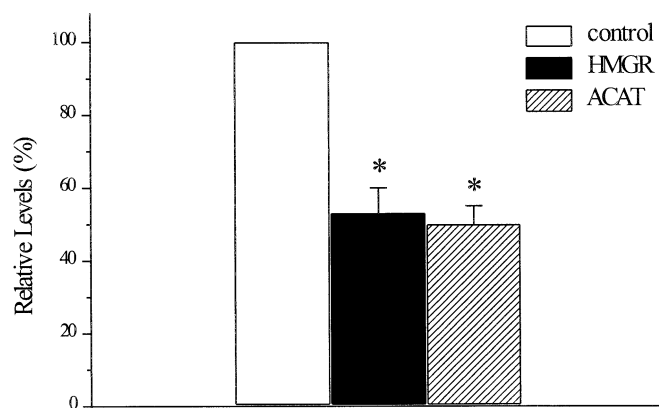


Fig. 3. Effects of taxifolin on HMGR and ACAT activity. HepG2 cells were treated with and without 200 μM taxifolin for a total of 24 h. HMGR activity was assayed in permeabilized cells in the presence and absence of taxifolin by [^{14}C]HMG-CoA labeling. The mevalonolactone formed was extracted, separated by TLC, and quantitated by scintillation counting. ACAT activity was determined in situ by [^{14}C]oleic acid labeling. The CE formed was extracted and separated by TLC, and the formation of CE was measured by scintillation counting. The results are expressed as a percentage of control (set as 100%). Values represent the mean \pm SD of three independent experiments in duplicate. * $P < 0.05$ versus control.

terol esterification by $53 \pm 2\%$ ($P < 0.05$, $n = 3$), suggesting that taxifolin may also limit ACAT activity. However, we cannot rule out the possibility that the decreased HMGR activity observed may have led to a decrease in esterified cholesterol due to lower levels of substrate.

Taxifolin also inhibits the synthesis and secretion of TAG and phospholipids

To evaluate further the effects of taxifolin on the synthesis and secretion of TAG and phospholipids, HepG2 cells were labeled with [^{14}C]glycerol with and without 200 μM taxifolin for a total of 24 h. As summarized in Fig. 4, taxifolin showed an inhibitory effect on the accumulation of TAG ($59 \pm 1\%$, $P < 0.05$, $n = 3$) and phospholipids ($15 \pm 2\%$, $P < 0.05$, $n = 3$) in the cell. Similar inhibitory effects on the secretion of TAG ($68 \pm 3\%$, $P < 0.05$, $n = 3$), with a more pronounced inhibition of phospholipid secretion ($57 \pm 1\%$, $P < 0.05$, $n = 3$), were also observed when compared with their synthesis level.

Taxifolin inhibits apoB and stimulates apoA-I secretion

A metabolic pulse-chase labeling experiment was performed to assess the fate of nascent apoB and apoA-I in untreated control and taxifolin-treated cell cultures. Cells were pulsed with [^{35}S]methionine/cysteine for 10 min, and chased with excess cold methionine/cysteine medium \pm taxifolin for up to 120 or 180 min. Aliquots of the intracellular and extracellular fractions were collected at various time points and analyzed. A preliminary experiment showed a 10- and 20-min delay in reaching peak incorporation of [^{35}S]methionine/cysteine into full-length apoA-I and apoB, respectively (data not shown).

Figure 5A is a fluorograph showing the amount of apoB

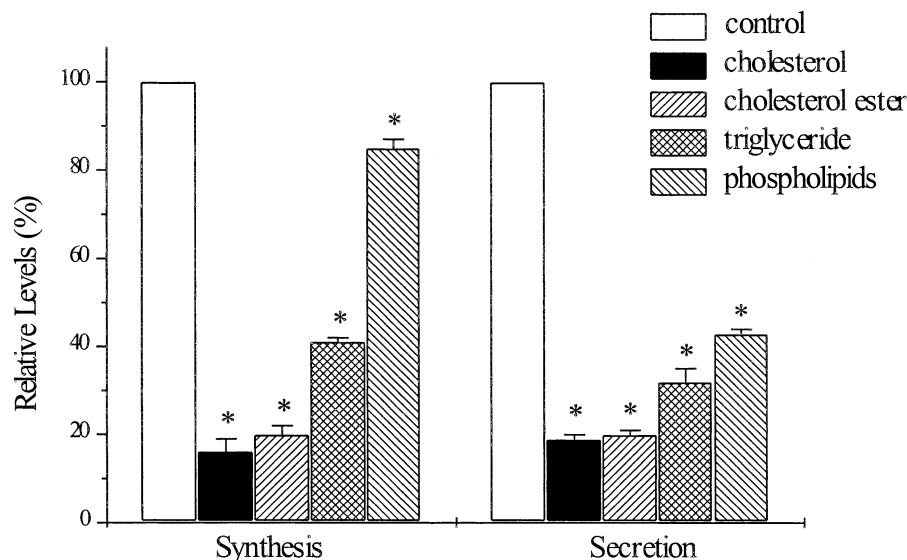


Fig. 4. Effects of taxifolin on lipid synthesis and secretion. In assessing lipid synthesis and secretion, cells were grown and treated with and without 200 μ M taxifolin for 18 h and then labeled for 6 h with [14 C]acetate (5 μ Ci/ml) or [14 C]glycerol (2.5 μ Ci/ml) \pm taxifolin. The amount of cellular and secreted lipids was determined by TLC and scintillation counting. The results are expressed as a percentage of control (set as 100%). Values represent the mean \pm SD of three independent experiments performed in duplicate. * $P < 0.05$ versus control.

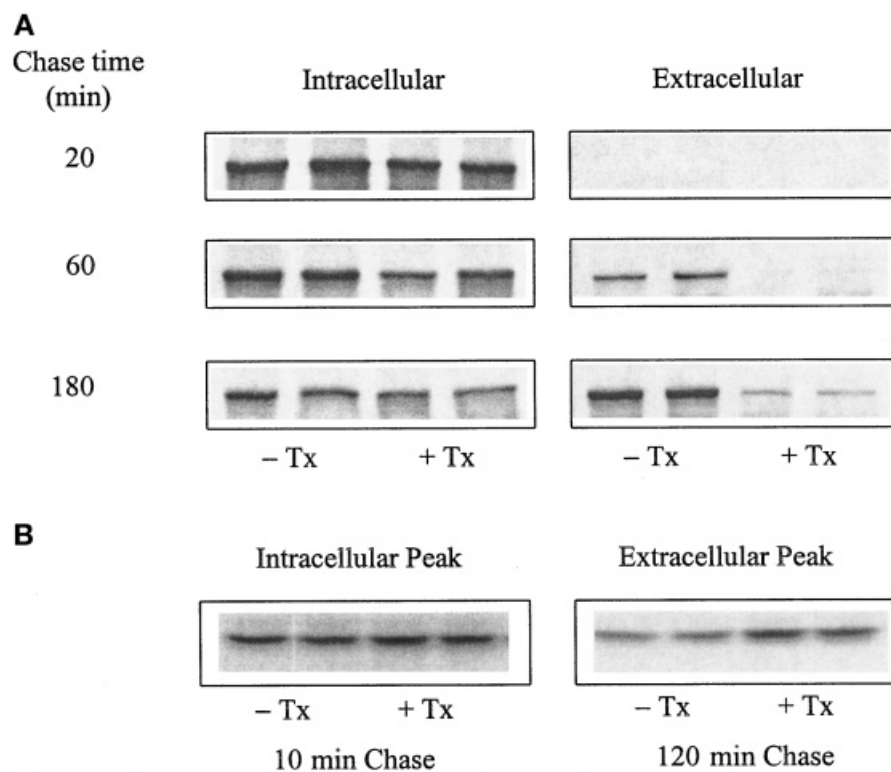


Fig. 5. Pulse-chase labeling of HepG2 cells. HepG2 cells were grown and treated with and without taxifolin (Tx) in SF-RPMI for 24 h. The cells were then pulsed for 10 min with [35 S]methionine/cysteine, washed, and chased with unlabeled methionine/cysteine for up to 120 or 180 min in the presence or absence of taxifolin. The labeled media (extracellular fraction) and cells (intracellular fraction) were collected at the end of each chase time and used for immunoprecipitation of apoB or apoA-I. The immunoprecipitates were analyzed by SDS-PAGE and fluorography, and apoB/apoA-I radioactivity was quantified by cutting and scintillation counting of the apoB/apoA-I bands. (A) Representative fluorograph showing the signals corresponding to apoB in both the intracellular and extracellular fractions at the various chase times. (B) Representative fluorograph showing the signals corresponding to apoA-I in both the intracellular and extracellular peak fractions. The experiment was performed three times in duplicate and the results were pooled, with standard deviations indicated in Fig. 6.

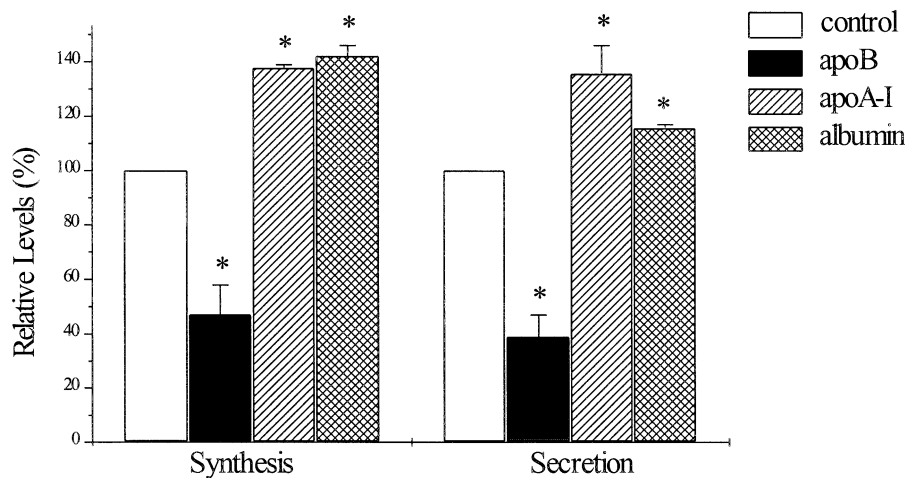


Fig. 6. Effects of taxifolin on the synthesis and secretion of ^{35}S -labeled apoB, apoA-I, and albumin during a 120- or 180-min chase. HepG2 cells were grown, treated, and pulse-chased as described in Fig 5. The intracellular fraction was collected at the beginning of the chase (peak incorporation), whereas the extracellular fraction was collected at the end of the chase period (peak secretion). The samples were subjected to immunoprecipitation of apoB, apoA-I, and albumin. Bar graph summarizes the data on apoB, apoA-I, and albumin synthesis and secretion relative to control (set as 100%). Data represent means \pm SD of three independent experiments performed in duplicate. * $P < 0.05$ versus control.

synthesized and secreted by HepG2 cells in the presence and absence of taxifolin in a typical pulse-chase experiment (reproduced in two other independent experiments; results pooled in **Fig. 6**). A 64% decrease in the incorporation of [^{35}S]methionine/cysteine into immunoprecipitable apoB was apparent with taxifolin at the 20-min chase time (representing peak incorporation). When the radioactivity was further chased, a gradual reduction in the intracellular labeled apoB was noted with a simultaneous increase in secreted labeled apoB. In the presence of taxifolin, a significant reduction in apoB secretion was noted (70%) when compared with the untreated control cells at the 180-min chase time (representing peak secretion). This effect on secretion with taxifolin was comparable to that of synthesis, indicating that the reduction in apoB secretion may be due to a decrease in newly synthesized apoB, possibly through a transcriptional and/or translational mechanism(s).

In parallel, the amount of apoA-I secreted in the presence and absence of taxifolin was also analyzed (**Fig. 5B**). Interestingly, a 35% increase in apoA-I secretion in taxifolin-treated cells was observed when compared with untreated control. A similar increase was noted for its synthesis rate (38%; reproduced in two other independent experiment; results pooled in **Fig. 6**). The increase in apoA-I secretion suggests that the inhibitory effect of taxifolin on apoB secretion is specific and that taxifolin may have benefits related to high density lipoprotein (HDL) metabolism.

As another control, the fate of nascent albumin synthesis and secretion were also examined during the chase. Results from three independent experiments performed in duplicate are summarized in **Fig. 6**. Measurement of albumin was done by immunoprecipitation, SDS-PAGE, and fluorography. A consistent increase in the synthesis of albumin ($42 \pm 4\%$, $P < 0.05$, $n = 3$) was observed at the beginning of the chase. The increase in synthesis corre-

sponded to an increase in albumin secretion ($16 \pm 1\%$, $P < 0.05$, $n = 3$) at the end of the chase. This further suggests that the inhibitory effect of taxifolin on apoB secretion is indeed specific. The increase in albumin and apoA-I syntheses is interesting and may reflect a global transcriptional effect. This is compatible with the mechanism of action of silybin, a flavolignan containing a taxifolin moiety, which can influence RNA polymerase activity (18).

Taxifolin has no effect on apoB and apoA-I mRNA steady-state levels

Our previous experiment indicated that the effect on apoB and apoA-I secretion appeared to be exerted at the synthesis level of these apolipoproteins. To address whether their transcription rate may be involved, a sensitive RNase protection/solution hybridization assay was used to measure apoB and apoA-I mRNA steady-state levels. Under our conditions (200 μM taxifolin, 24 h), hepatic apoB and apoA-I mRNA abundances remained essentially unchanged when compared with the untreated control cells (**Table 1**). This rules out transcriptional effects and indicates that other regulatory mechanism(s) are being exerted.

Taxifolin decreases the secretion of apoB into LDL-like particles

To examine the distribution of secreted apoB-containing lipoprotein (apoB-Lp) with taxifolin, the density of secreted apoB-Lp particles in HepG2 cells was investigated by sucrose gradient ultracentrifugation. ApoB-Lp secreted by cells was observed to have a similar density to that of human plasma LDL, as has been observed in several studies [reviewed in ref. (19)]. As depicted in **Fig. 7**, taxifolin markedly reduced the amount of secreted apoB-Lp with a slight shift in the density profile

TABLE 1. ApoB and apoA-I mRNA abundances in control and taxifolin-treated cells^a

Control	ApoB mRNA ^b		P	Control	ApoA-I mRNA ^c		P
	Taxifolin				Taxifolin		
1,068.79 ± 97.67	1,172.33 ± 152	0.289		71.43 ± 4.5	70.58 ± 4.51	0.449	

^a ApoB and apoA-I mRNA levels are expressed as picograms of mRNA per microgram of total RNA.

^b Values represent the mean ± SEM of three experiments performed in duplicate.

^c Values represent the mean ± SEM of two experiments performed in duplicate.

of secreted particles (fractions 2–5 represent dense HDL-like apoB-Lp, whereas fraction 6–12 represent LDL-like apoB-Lp). This suggests that taxifolin and perturbed lipid synthesis can partially block the secretion of apoB-Lp particles. Whether taxifolin affects the assembly process of apoB-Lp particles will require further investigation.

Taxifolin stimulates apoB degradation via a DTT-sensitive, ALLN-insensitive proteolytic pathway

Intracellular proteasomal degradation of apoB has been well described as the major regulatory mechanism of action in apoB-Lp secretion (19, 20). To determine whether ALLN-sensitive proteasome is involved in the taxifolin-induced changes in apoB secretion, pulse-chase experiments were performed. Taxifolin-treated and untreated control cells were pretreated for 10 min with and without ALLN (20 µg/ml) and taxifolin. After a 10-min pulse, the cells were chased for 5 min and harvested for apoB immunoprecipitation. As shown in Fig. 8, addition of ALLN to the untreated control cells (lanes 9 and 10) failed to accumulate apoB intracellularly, indicating a probable rapid cotranslational degradation of apoB. In

the presence of taxifolin (Fig. 8, lanes 11 and 12), apoB cellular content was further reduced and was not blocked by ALLN. This suggests that other protease(s) may be responsible for apoB degradation.

Benoist, Nicodeme, and Grand-Perret (21) have suggested that another protease, a DTT-sensitive protease, may be involved in the early stages of apoB degradation. Hence, we examined the possibility that such a protease could mediate the effect of taxifolin on apoB secretion. We used a DTT protocol as described by Shelness and Thornburg (22). Taxifolin-treated and untreated cells were first pretreated for 1 min with and without DTT (2 mM) in the presence and absence of taxifolin. After a 10-min pulse, the cells were chased for 5 min and harvested for apoB immunoprecipitation. Interestingly, as shown in Fig. 8, addition of DTT to the taxifolin-treated cells (lanes 7 and 8) increased apoB cellular content comparable to the untreated DTT control cells (lanes 5 and 6). Despite a reduction in protein synthesis with DTT as has been observed by others (21, 22), the effect of taxifolin on early apoB degradation was virtually prevented. This suggests that a putative DTT-sensitive protease may be involved.

Taxifolin partially inhibits the stimulatory effect of oleate and 25-hydroxycholesterol on apoB secretion

To investigate the effect of taxifolin on apoB secretion under lipid-rich conditions, both oleate (360 µM complexed to BSA) and 25-hydroxycholesterol (10 µg/ml dissolved in ethanol) were added overnight in the presence or absence of taxifolin (200 µM). Figures 9A and B demonstrate the amount of apoB secreted over a 2-h chase in the presence and absence of taxifolin. Experiments with oleate were conducted in the presence of medium plus BSA, medium plus BSA/taxifolin, medium plus BSA/oleate, and medium plus BSA/oleate/taxifolin. Treatment with oleate showed a 4-fold stimulation of apoB secretion compared with BSA-untreated cells. Coincubation with both oleate and taxifolin resulted in a lower stimulation of apoB secretion (39% decrease vs. oleate-treated cells; reproduced in one other independent experiment). Similarly, 25-hydroxycholesterol also resulted in a significant elevation of apoB secretion when compared with the ethanol-treated control cells (2-fold stimulation). In contrast, the effect of 25-hydroxycholesterol with taxifolin was more pronounced than with oleate, as taxifolin was shown to markedly reverse the stimulatory effect of 25-hydroxycholesterol on apoB secretion (75% vs. 25-hydroxycholesterol-treated cells; reproduced in one other independent experiment).

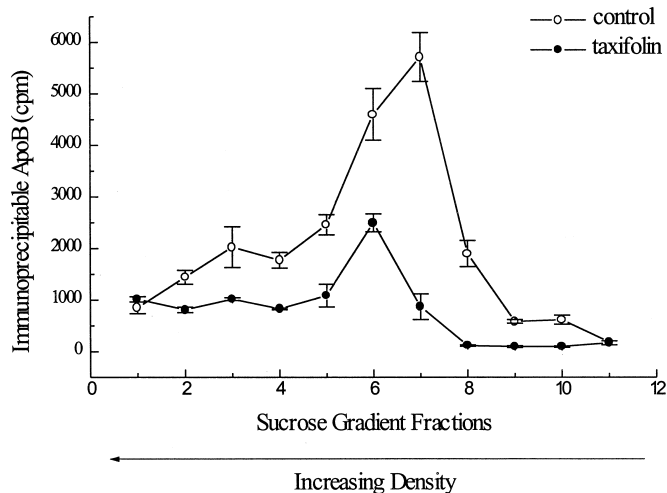


Fig. 7. Density distribution of secreted apoB-Lp in control and taxifolin-treated cells. Treated and untreated HepG2 cells were grown, pulsed, and chased as described in Fig. 5. The extracellular fraction was collected at the end of the chase period (2 h) and subjected to sucrose gradient ultracentrifugation. 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 and scintillation counting of the apoB band. Data represent means ± SD of two independent experiments performed in duplicate.

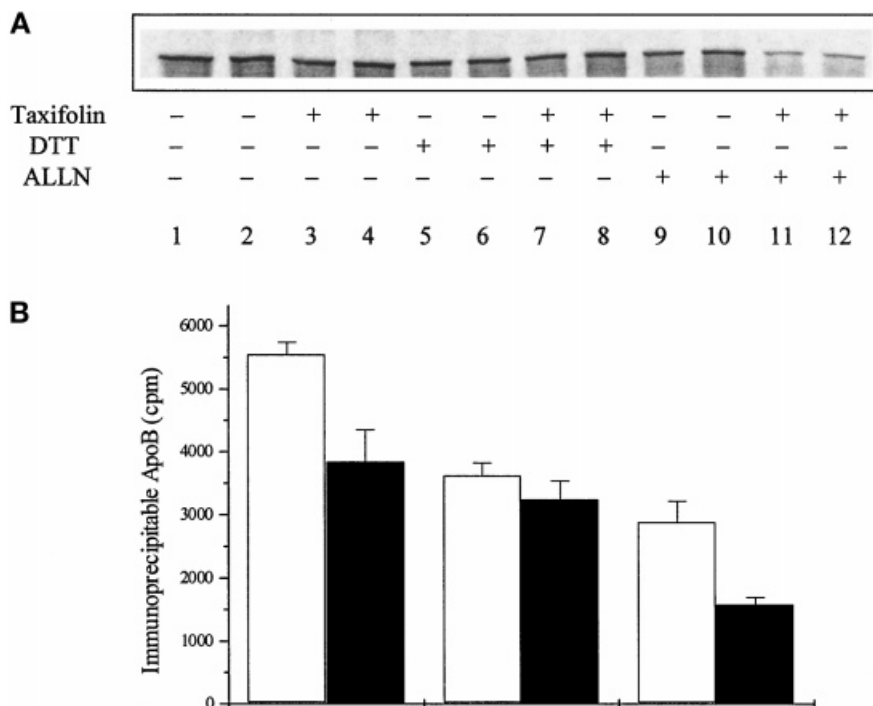


Fig. 8. Effect of taxifolin on early apoB degradation. Treated and untreated control cells were first pre-treated with or without 200 μ M taxifolin either in the presence or absence of ALLN (20 μ g/ml) for 10 min or DTT (2 mM) for 1 min. A 10-min pulse was followed by a 5-min chase in the presence and absence of taxifolin/ALLN/DTT. Cells were harvested and immunoprecipitated for apoB. The samples were subjected to SDS-PAGE, fluorography, and quantification. (A) Representative fluorograph showing the signals corresponding to apoB in the intracellular fractions at the 5-min chase. (B) Bar graph showing the mean of duplicate values \pm SD of the experiment in (A). Results are given as counts per minute per well. Data are typical of an experiment performed twice in duplicate.

DISCUSSION

Taxifolin is a plant flavonoid occurring in the fruit of *S. marianum*. This fruit, also known as “milk thistle,” has been used since ancient times as a medicinal plant for the treatment of liver diseases in traditional European and Asiatic medicine [reviewed in ref. (18)]. The extract, known as silymarin, contains a mixture of flavonoids, namely silybin, along with other minor components such as taxifolin. Because of taxifolin’s structural homology to silybin, a

known HMGCR inhibitor, we proposed that taxifolin be studied as a potential hypocholesterolemic agent.

The present study provides evidence that taxifolin is capable of influencing lipid and apolipoprotein production. Initial studies examined the effects of taxifolin on lipid synthesis and secretion in HepG2 cells. Our results confirmed that taxifolin is able to decrease [14 C]acetate incorporation into cholesterol in a dose- and time-dependent manner. Interestingly, acetate incorporation was inhibited by as much as 86% at 200 μ M, a concentration at which

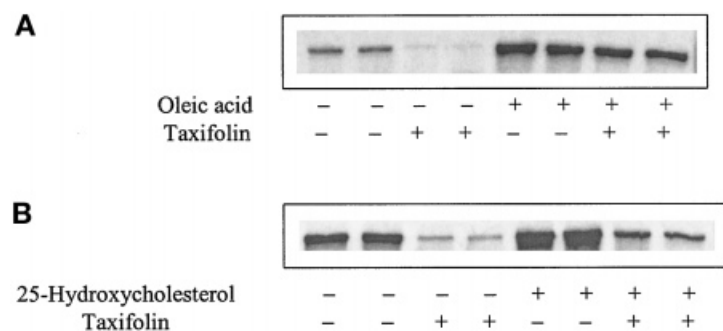


Fig. 9. Effect of taxifolin on apoB secretion in oleate- and 25-hydroxycholesterol-treated cells. HepG2 cells were treated with 200 μ M taxifolin for 24 h in the presence or absence of BSA-bound oleate (360 μ M) or 25-hydroxycholesterol (10 μ g/ml). Cells were pulsed and chased for 2 h. Medium was collected and apoB was immunoprecipitated, followed by SDS-PAGE and fluorography. Quantification was done by scintillation counting of the apoB band. (A) Representative fluorograph showing the amount of radiolabeled apoB secreted from cells treated with medium plus BSA, medium plus BSA/taxifolin, medium plus BSA/oleate, and medium plus BSA/oleate/taxifolin. (B) Representative fluorograph showing the amount of radiolabeled apoB secreted from cells treated with medium, medium plus taxifolin, medium plus 25-hydroxycholesterol, and medium plus 25-hydroxycholesterol/taxifolin. Data are typical of an experiment performed twice in duplicate.

cell viability was not altered. Consequently, this concentration was chosen in the following experiments and represents a pharmacological dose. Under this condition, taxifolin was shown to equally inhibit both free cholesterol and CE synthesis. Moreover, using [¹⁴C]glycerol incorporation studies, TAG and phospholipid synthesis was also found to be significantly reduced. These results with lipids are, on one hand, consistent with that from silybin and silymarin. Studies by Nassuato et al. (9), Montanini et al. (23), and Petronelli et al. (24) showed that silybin and silymarin decreased hepatic synthesis of cholesterol, phospholipids, and TAG. On the other hand, our results are in contrast with the citrus flavonoids hesperetin and naringenin, which were shown to have little inhibitory effect and, in some cases, a stimulatory effect on free cholesterol, TAG, and phospholipid synthesis in HepG2 cells (7, 8). The discrepancy in results may stem from structural differences in the flavonoid molecules. Accordingly, taxifolin appears to be more effective than hesperetin and naringenin in reducing lipid synthesis. It is possible that taxifolin may have a more global transcriptional regulation in lipogenesis with such proteins as the sterol regulatory element-binding protein. Secretion of lipids into the culture medium was also investigated in taxifolin-treated cells. Our results indicated a similar decrease in free cholesterol, CE, and TAG, with a more pronounced inhibition of phospholipid secretion compared with the effect observed on phospholipid synthesis.

In elucidating the mechanism of action on cholesterol synthesis, we examined the effect of taxifolin on HMGCR activity, a key enzyme in cholesterol biosynthesis. Nassuato et al. (9) demonstrated a dose-dependent inhibition of HMGCR by silybin. Our results showed that the taxifolin moiety of silybin also inhibited HMGCR activity. Although the level at which taxifolin may be exerting its effect on HMGCR gene expression remained unknown, the data did yield information about the level at which taxifolin influences hepatic lipid synthesis. Thus, taxifolin is suggested to behave like a statin drug. Unlike these common cholesterol-lowering drugs, taxifolin may be hepatoprotective and exhibit beneficial antioxidant characteristics (25, 26).

Evidence that flavonoids may also inhibit ACAT activity and cholesterol esterification has been reported by a number of studies (7, 10, 27, 28). Hence, we examined whether taxifolin shares this property. We have shown that taxifolin indeed reduced the incorporation of [¹⁴C]oleate into cellular CE in situ, suggesting ACAT activity may also be involved. However, as previously noted, we cannot completely rule in ACAT activity because the decreased HMGCR activity may have led to the decreased esterified cholesterol due to the lower levels of substrate.

Because apolipoproteins play an important role in cholesterol transport, we continued our study by investigating the effects of taxifolin on apoA-I and apoB synthesis and secretion. Although apoA-I is associated mainly with HDL and is involved in the reverse cholesterol transport pathway, apoB is associated with LDL and is said to be atherogenic because it delivers cholesterol to the cell. Using pulse-chase experiments, the rates of synthesis and secre-

tion were estimated. We found the incorporation of [³⁵S]methionine/cysteine to be markedly decreased in cellular apoB and increased in cellular apoA-I, suggesting that changes in the rate of apolipoprotein synthesis may be involved. The changes in the amount of cellular apoA-I and apoB in the presence of taxifolin translated into a similar effect on apoA-I and apoB levels appearing in the medium. Because synthesis was found to be affected, the possibility that taxifolin altered transcription and/or translation of apoB and apoA-I was not excluded. However, in the case of apoB, these mechanisms of action are unlikely because most reports have indicated that changes in apoB secretion are primarily regulated co- and posttranslationally through intracellular degradation [reviewed in ref. (19)]. According to this model, newly synthesized apoB associated with the cytosolic side of the ER membrane is degraded cotranslationally by the proteasome (20), whereas fully translocated apoB is thought to be degraded either posttranslationally by an ER-localized cysteine protease (29) or translocated back out to the cytosol cotranslationally for proteasomal degradation (30). In addition, there is indication that a DTT-sensitive protease may be involved in the early stages of apoB degradation (21, 31). Our results are consistent with early degradation represented by a significant decrease in the intracellular concentration of newly synthesized apoB at the beginning of the chase in our metabolic pulse-chase labeling experiments. Although the apoB mRNA level was found to remain essentially unchanged, evidence of cotranslational control was provided by our protease data. Interestingly, our data support the hypothesis that taxifolin is involved at an early stage of apoB processing and that DTT can reverse the effect of taxifolin on apoB secretion. In contrast, the lack of response to the proteasome inhibitor ALLN argues against involvement of the proteasome in taxifolin-induced changes in apoB secretion. Taken together, the mechanism of taxifolin-mediated lipoprotein assembly with apoB appears to involve a DTT-sensitive, ALLN-insensitive degradation pathway. These results are compatible with Benoist, Nicodeme, and Grand-Perret (21), whose microsomal triglyceride transfer protein inhibitor was shown to act rapidly through a DTT-sensitive, ALLN-insensitive proteolytic pathway. Also, this is compatible with the results from Borradaile, Carroll, and Kurowska (8) on the inhibitory effects of naringenin and hesperetin on apoB secretion. As observed by these investigators, coincubation with the proteasome inhibitor MG132 failed to increase apoB secretion, indicating that proteasomal degradation was not involved. The exact nature and role of our DTT-sensitive protease in apoB degradation remain to be elucidated.

The finding of reduced apoB secretion of cells incubated with taxifolin is also supported by the analysis of the lipoprotein fraction distribution, which showed a reduced accumulation of labeled LDL-like apoB in the medium of cells incubated with taxifolin. No major shift in the distribution of secreted lipoproteins was observed when compared with untreated control cells. Together, these results indicated that taxifolin decreased the number of apoB-Lp

secreted without a major change in the density of the assembled lipoproteins.

The effect on apoB synthesis and secretion was found to be specific when compared with apoA-I and albumin. Both albumin and apoA-I synthesis and secretion were shown to be stimulated. This effect is interesting and may reflect a global transcriptional effect. This is compatible with the mechanism of action of silybin, the flavolignan containing a taxifolin moiety, which can influence RNA polymerase activity thought to be important in the repair phase of liver damage (18). However, the apoA-I transcription rate remained essentially unchanged with taxifolin, suggesting that other mechanism(s) may be involved. Nevertheless, silybin, as well as silymarin, were shown in hypercholesterolemic rats to increase HDL-cholesterol levels (11), which is compatible with our findings on apoA-I. Thus, taxifolin appears to have benefits related to HDL metabolism.

The assembly of apoB-Lp particles is a complex process that requires the coordinated synthesis and assembly of apoB, TAG, CE, phospholipids, and other components. Because lipid availability is a major determining factor in the assembly and secretion of apoB-Lp, we studied the effects of exogenous lipids on the ability of taxifolin to reduce apoB secretion in HepG2 cells. The addition of oleate and 25-hydroxycholesterol has been previously shown to stimulate the secretion of apoB in these cells (32, 33). Oleate presumably functions to enhance TAG synthesis, whereas 25-hydroxycholesterol increases CE content. Results of these experiments showed a remarkable increase in apoB secretion in oleate- and 25-hydroxycholesterol-treated cells. On addition of taxifolin with oleate, a moderate reduction in apoB secretion was observed. Interestingly, however, the effects were more pronounced with 25-hydroxycholesterol, which markedly diminished the secretion of apoB. The fact that taxifolin reversed the effect of each lipid differently is intriguing and may reflect that CE content, rather than TAG, is primarily associated with changes in apoB secretion under taxifolin treatment. Thus, taxifolin may be exerting its apoB-Lp-lowering action by limiting CE availability essential for the assembly of apoB-Lp.

In summary, the data in this report indicate that taxifolin inhibited the synthesis and secretion of a number of lipids, in addition to decreasing apoB and increasing apoA-I secretion. This supports the theory that taxifolin, the moiety of the flavolignan silybin, may represent a potentially important method of controlling atherogenesis. This may also account for the superior hypocholesterolemic activity of silymarin relative to silybin. The suggested statin-like activity of taxifolin may lead to an alternative nutraceutical agent with combined hypocholesterolemic and antioxidant properties. ■

The investigation was supported by a Research Centers of Minority Institutions (RCMI) award, P20 RR/AI 11091, from the National Center for Research Resources, National Institutes of Health.

Manuscript received 18 May 2000 and in revised form 24 July 2000.

REFERENCES

- Hollman, P. C., M. G. Hertgo, and M. B. Katan. 1996. Role of dietary flavonoids in protection against cancer and coronary heart disease. *Biochem. Soc. Trans.* **24**: 785–789.
- De Whalley, C. V., S. M. Rankin, J. R. Houlst, W. Jessup, and D. S. Leake. 1990. Flavonoids inhibit the antioxidative modification of low density lipoproteins. *Biochem. Pharmacol.* **39**: 1743–1749.
- Hayek, T., B. Fuhrman, J. Vaya, M. Rosenblat, P. Belinky, R. Coleman, A. Elis, and M. Aviram. 1997. Reduced progression of atherosclerosis in apoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol.* **17**: 2744–2752.
- Choi, J. S., T. Yokozawa, and H. Oura. 1991. Antiatherogenic effects of flavonoids from *Prunus Davidiana*. *J. Nat. Prod.* **54**: 218–224.
- Jahromi, M. A. F., and A. B. Ray. 1993. Antiatherogenic effect of flavonoids from *Pterocarpus marsupium*. *J. Nat. Prod.* **56**: 989–994.
- Anderson, J. W., B. M. Johnstone, and M. E. Cook-Newell. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **333**: 276–282.
- Wilcox, L. J., N. M. Borradaile, E. M. Kurowska, D. E. Telford, and M. W. Huff. 1998. Naringenin, a citrus flavonoid, markedly decreases apoB secretion in HepG2 cells and inhibits acyl CoA:cholesterol acyltransferase. *Circulation.* **98**: I-537 (Abstract).
- Borradaile, N. M., K. K. Carroll, and E. M. Kurowska. 1999. Regulation of HepG2 cell apolipoprotein B metabolism by the citrus flavanones hesperetin and naringenin. *Lipids.* **34**: 591–598.
- Nassuato, G., R. M. Iemmo, M. Strazzabosco, F. Lirussi, R. Deana, M. A. Francesconi, M. Muraca, D. Passera, A. Fragasso, R. Orlando, G. Csomos, and L. Okolicsanyi. 1991. Effect of silybinin on biliary lipid composition: experimental and clinical study. *J. Hepatol.* **12**: 290–295.
- Bok, S-H., S-H. Lee, Y-B. Park, K-H. Bae, K-H. Son, T-S. Jeong, and M-S. Choi. 1999. Plasma and hepatic cholesterol and hepatic activities of 3-hydroxy-3-methylglutaryl-CoA reductase and acyl CoA:cholesterol transferase are lower in rats fed citrus peel extract or a mixture of citrus bioflavonoids. *J. Nutr.* **129**: 1182–1185.
- Krecman, V., N. Skottova, D. Walterova, J. Ulrichova, and V. Simanek. 1998. Silymarin inhibits the development of diet-induced hypercholesterolemia in rats. *Planta Med.* **64**: 138–142.
- Goldstein, J., S. Basu, and M. Brown. 1983. Assay of cholesterol ester formation in cultured cells. *Methods Enzymol.* **98**: 257–260.
- Leonard, D. A., and H. W. Chen. 1987. ATP-dependent degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in permeabilized cells. *J. Biol. Chem.* **262**: 7914–7919.
- Therault, A., Q. Wang, A. Gapor, and K. Adeli. 1999. Effects of γ -tocotrienol on apolipoprotein B synthesis, degradation, and secretion in HepG2 cells. *Arterioscler. Thromb. Vasc. Biol.* **19**: 704–712.
- Azrolan, N., and J. L. Breslow. 1990. A solution hybridization/RNase protection assay with riboprobes to determine absolute levels of apoB, apoA-I, and E mRNA in human hepatoma cell lines. *J. Lipid Res.* **31**: 1141–1146.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Trzasko, J. M., R. L. Magolda, M. F. Favata, R. T. Fischer, P. R. Johnson, H. W. Chen, S. K. Soo, D. A. Leonard, and J. L. Gaylor. 1993. Modulation of 3-hydroxy-3-methylglutaryl-CoA reductase by 15 α -fluorolanost-7-en-3 β -ol. *J. Biol. Chem.* **268**: 22591–22599.
- Morazzoni, P., and E. Bombardelli. 1995. *Silybum marianum* (*Cardus marianus*). *Fitoterapia.* **66**: 6–42.
- Olofsson, S-O., A. Lennart, and J. Boren. 1999. The assembly and secretion of apolipoprotein B-containing lipoproteins. *Curr. Opin. Lipidol.* **10**: 341–346.
- Yeung, S. J., S. H. Chen, and L. Chan. 1996. Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein B. *Biochemistry.* **35**: 13843–13848.
- Benoist, F., E. Nicodeme, and T. Grand-Perret. 1996. Microsomal triacylglycerol transfer protein prevents presecretory degradation of apolipoprotein B: A dithiothreitol-sensitive protease is involved. *Eur. J. Biochem.* **240**: 713–720.
- Shelness, G. S., and J. T. Thornburg. 1996. Role of intramolecular disulfide bond formation in the assembly and secretion of apolipoprotein B-100-containing lipoproteins. *J. Lipid Res.* **37**: 408–419.
- Montanini, I., F. Castigli, G. Arienti, and G. Porcellati. 1977. The

effect of silybin on liver phospholipid synthesis in the rat in vivo. *Farmacol. Ed. Sci.* **32**: 141–146.

24. Petronelli, A., E. Roda, M. Briganti, A. M. Morselli-Labate, and L. Barbara. 1981. Effect of the administration of silymarin on serum lipid levels. *Clin. Ter.* **99**: 471–482.
25. Feher, J., A. Cornides, J. Pal, I. Lang, and G. Csomos. 1989. Liver cell protection in toxic liver lesion. *Acta Physiol. Hung.* **73**: 285.
26. Kandaswami, C., and E. Middleton. 1994. Free radical scavenging and antioxidant activity of plant flavonoids. In *Free Radicals in Diagnostic Medicine*. D. Armstrong, editor. Plenum Press, New York. 351–376.
27. Yotsumoto, H., T. Yanagita, K. Yamamoto, Y. Ogawa, J. Y. Cha, and Y. Mori. 1997. Inhibitory effects of oren-gedoku-to and its components on cholesterol ester synthesis in cultured human hepatocyte HepG2 cells: evidence from the cultured HepG2 cells and in vitro assay of ACAT. *Planta Med.* **63**: 141–145.
28. Wilcox, L. J., L. E. De Dreu, N. Borradaile, and M. W. Huff. 1999. The soy phytoestrogen, genistein and daidzein, decrease apolipoprotein B secretion by HepG2 cells. *Circulation.* **100**: I-109 (Abstract).
29. Adeli, K., J. Macri, A. Mohammadi, M. Kito, R. Urade, and D. Cavallo. 1997. Apolipoprotein B is intracellularly associated with an ER-60 protease homologue in HepG2 cells. *J. Biol. Chem.* **272**: 22489–22494.
30. Liao, W., S-C. J. Yeung, and L. Chan. 1998. Proteasome-mediated degradation of apolipoprotein B targets both nascent peptides cotranslationally before translocation and full-length apolipoprotein B after translocation into the endoplasmic reticulum. *J. Biol. Chem.* **273**: 27225–27230.
31. Wu, X., N. Sakata, K. M. Lele, M. Zhou, H. Jiang, and H. N. Ginsberg. 1997. A two-site model for apoB degradation in HepG2 cells. *J. Biol. Chem.* **266**: 5080–5086.
32. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266**: 5080–5086.
33. Dashti, N. 1992. The effect of low density lipoproteins, cholesterol, and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. *J. Biol. Chem.* **267**: 7160–7169.