HepG2 cell LDL receptor activity and the accumulation of apolipoprotein B and E in response to docosahexaenoic acid and cholesterol

Mary Sorci-Thomas,[1] Cynthia L. Hendricks, and Mary W. Kearns

Department of Comparative Medicine and the Arteriosclerosis Research Center, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27157

Abstract In the present study, the accumulation of apolipoproteins (apo) A-I, B, and E in culture medium was measured after 0, 3, 6, 12, and 24 h of incubation with 150 μM docosahexaenoic acid complexed to 75 μM bovine serum albumin (BSA-22:6), either in the presence or absence of 50 μg/ml cholesterol and 4 μg/ml 25-hydroxycholesterol (C/25-OH). HepG2 cells incubated with BSA + C/25-OH for 24 h accumulated approximately 2.0-fold greater apoE and B as compared to BSA-treated cells. Moreover, HepG2 cell apoB accumulation after 24 h of BSA-22:6 treatment was approximately 2.0-fold greater than apoB accumulation from cells treated with BSA alone. When BSA-22:6 and C/25-OH were both included in the incubation, apoB accumulation was approximately 5.0-fold greater than BSA-treated cells. Comparative studies using BSA-18:1 were carried out for 24 h and showed similar levels of apoA-I, B, and E accumulation in culture medium as compared to BSA-22:6-treated cells. In addition, apoA-I, B, and E mRNA abundance were found to be unaffected by type of fatty acid treatment or length of incubation, averaging 48.2 ± 7.5, 222 ± 33.6, and 17.1 ± 0.7 pg mRNA/μg RNA (mean ± SEM), respectively. As the accumulation of apoB and apoE in culture medium may be modified by HepG2 cell LDL receptor expression, LDL receptor mRNA abundance and LDL receptor activity were quantified at various times over the course of the study. By 6 h of BSA + C/25-OH treatment, LDL receptor mRNA was reduced approximately 2.3-fold, while receptor activity was reduced approximately 1.5-fold, as compared to BSA controls. In an experiment designed to determine uptake of HepG2 cell lipoproteins, ^{3}H-labeled apoB-containing lipoproteins derived from HepG2 cells were prepared. The ^{3}H-labeled lipoproteins were 1.25-fold more likely to be removed from the media of HepG2 cells treated with BSA than from cells treated with BSA + C/25-OH. From these results, we postulate that HepG2 cell LDL receptor activity mediates the removal of apoB, E-containing lipoproteins from culture medium and contributes to the lower accumulation of apoB and E observed in culture medium from cells treated with BSA as compared to cells treated with C/25-OH.—**Sorci-Thomas, M., C. L. Hendricks, and M. W. Kearns.** HepG2 cell LDL receptor activity and the accumulation of apolipoprotein B and E in response to docosahexaenoic acid and cholesterol. *J. Lipid Res.* 1992. **33:** 1147–1156.

Supplementary key words apolipoprotein • low density lipoprotein receptor • 25-hydroxycholesterol • mRNA

Mechanisms that regulate triglyceride-rich apolipoprotein (apo) B, E-containing particle synthesis and catabolism by the liver are of considerable importance as plasma LDL concentrations correlate with the risk of developing coronary heart disease. Humans and nonhuman primates, as well as cell culture model systems, have been used in the effort to study effects of fish oil on apolipoprotein synthesis. In human populations that consume oils rich in n–3 fatty acids, a lower incidence of cardiovascular disease is observed as compared to those consuming diets rich in saturated fat (1–3). In humans, dietary fish oils have a variety of effects on plasma lipoprotein fractions but in virtually every case reported fish oils reduce plasma triglyceride levels (for review see ref. 4), with much of the variability due to the duration of the study and dosage or type of dietary fat used. In nonhuman primates fed fish oil for periods longer than 5 months, the concentration of plasma cholesterol is significantly decreased as compared to levels in animals fed a lard diet. Consistently, fish oil-fed African green monkeys show decreases in HDL cholesterol, apoA-I, LDL cholesterol, and total plasma cholesterol concentrations, without significant decreases in plasma apoB concentrations, (for review see ref. 5). These reductions in total plasma cholesterol concentrations correlated with a lower incidence of coronary artery atherosclerosis (6) when compared to their lard-fed counterparts.

Fatty acid-mediated changes in triglyceride-rich apoB-containing particle synthesis have also been studied in rat liver and primary hepatocytes (7–10), and in human hepatoma cells (HepG2) (11–23). The fatty acid most exten-

Abbreviations: FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

[1]To whom correspondence should be addressed at: Department of Comparative Medicine, Bowman Gray School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157.

sively studied in HepG2 cells, oleic acid (18:1), has been shown to increase apoB accumulation in culture medium to a small or insignificant degree (12, 14–17), or 2.0- to 7.0-fold (13, 18–23). Fish oil-derived fatty acids such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) have been shown to decrease apoB-containing lipoprotein accumulation when compared to 18:1-treated cells (12, 13). While it is generally agreed that fatty acid effects on apoB accumulation are associated with little if any change in apoB mRNA abundance (12, 16, 18, 19), recent results suggest that fatty acid treatment may alter apoB secretion at a post-transcriptional level. Studies investigating the synthesis and secretion of apoB-containing lipoproteins in HepG2 cells indicate that the rate of apoB degradation (20, 24) determines the overall rate of intracellular apoB secretion. Furthermore, these studies have shown that treatment of HepG2 cells with 18:1 inhibits the intracellular degradation of apoB (20), resulting in a greater accumulation of apoB-containing particles.

The effects of fish oil on HepG2 cell apoB-containing particle catabolism have been less well studied, although expression and regulation of LDL receptors in HepG2 cells have been demonstrated (25–30). In one report by Wong and Nestel (13) eicosapentaenoic acid treatment was shown to reduce HepG2 cell radiolabeled LDL saturable binding as compared to oleic acid and control incubations; whereas the binding of triglyceride-rich lipoproteins containing chylomicron remnants was not affected by fish oil treatment.

We undertook the present study to establish a relationship between HepG2 cell apolipoprotein gene expression and LDL receptor activity in response to docosahexaenoic acid (22:6) and cholesterol treatment. These studies show that by 12 h of treatment both apoB and apoE accumulation were stimulated approximately 2.0-fold after treatment with BSA + cholesterol/25-hydroxycholesterol as compared to cells treated only with BSA. When BSA-22:6 + cholesterol/25-hydroxycholesterol were both included in the incubation, apoB accumulation was approximately 5.0-fold greater than in BSA-treated cells. In contrast, apoE accumulation was stimulated 1.8-fold in response to cholesterol/25-hydroxycholesterol and not by BSA-22:6 treatment. Another aspect of these studies was to determine the extent to which LDL receptor activity influences apolipoprotein accumulation. In studies designed to show that HepG2 cell LDL receptor activity alters the overall mass accumulation of apoB and E in culture medium with time, HepG2 cells removed 1.25-fold less HepG2 cell-derived ^{3}H-labeled apoB-containing lipoproteins when pretreated with BSA + cholesterol/25-hydroxycholesterol than BSA controls. From these results, we postulate that HepG2 cell LDL receptor activity mediates the removal of apoB, E-containing particles from the culture medium and contributes to the lower accumulation of B and E observed in the culture medium from cells treated with BSA as compared to cells treated with cholesterol/25-hydroxycholesterol.

MATERIALS AND METHODS

All radioactive nucleotides were purchased from Du Pont New England Nuclear; Na ^{125}I (carrier-free) was obtained from Amersham. Modification and restriction enzymes were purchased from GIBCO/BRL. Ultrapure nucleotides and S1 nuclease were purchased from Pharmacia LKB Biotechnology Inc. Cell culture medium and nutrients were obtained from Hazelton Dutchland, Inc. Fatty acid-free bovine serum albumin (BSA) fraction V and cholesterol (99 + % pure) were purchased from Sigma Chemical Co. Docosahexaenoic acid (22:6) was supplied by Nu-Chek-Prep and was found to be greater than 99% pure as determined by gas-liquid chromatography. 5-Cholesten-3β, 25-diol (25-hydroxycholesterol) (99 + %) pure was supplied from Steraloids Inc. Triglycerides were measured from sonicated cell pellets with an enzymatic reagent kit from Boehringer Mannheim Diagnostics.

HepG2 cell culture

Human hepatoma (HepG2) cells were maintained at 37°C in equilibration with 5% CO_2–95% air in 75-cm^2 flasks containing maintenance medium plus 10% fetal bovine serum (FBS). The maintenance media (MEM) consisted of minimal essential medium containing Earle's salts and nonessential amino acids supplemented with minimal essential medium vitamins and a final concentration of 2 mM L-glutamine and 1 μM sodium pyruvate. Subcultures of cells for use in experiments were obtained from a 1:4 split of confluent monolayers. Cells were seeded on 60-mm plates and grown to 85–90% confluence (approximately 1.6 • 10^{6}cells/dish) in MEM + FBS. Twenty four hours before the cells were used in an experiment, they were incubated in FBS-free MEM containing a final concentration of 0.5% BSA (75 μM). At the start of the experiment this medium was removed and the monolayer was gently washed 2 times with 136 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate, pH 7.3 (PBS), and cells were then incubated in FBS-free MEM supplemented with one of the following additions to give the final concentrations: 75 μM BSA; 150 μM 22:6 complexed to 75 μM BSA (BSA-22:6); 75 μM BSA and 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol; or 150 μM 22:6 complexed to 75 μM BSA and 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol. Preparation of BSA complexed with 22:6 or 18:1 was carried out according to previously published procedures (31). For studies involving cholesterol and 25-hydroxycholesterol, a stock solution containing 10.0 mg/ml cholesterol and 0.8 mg/ml 25-hydroxycholesterol was dissolved in ethanol and

5 μl/ml culture medium was used to obtain a final concentration of 50 μg/ml cholesterol and 4 μg/ml 25-hydroxycholesterol.

RNA isolation and quantification of mRNA

Immediately after removal of the culture medium, cells were collected from the dishes by addition of 0.5 ml of 0.25% trypsin solution. Cells were transferred to a 15-ml conical tube and the dishes were washed two additional times with 2 ml of cold PBS. Cells were pelleted by centrifuging at 2,000 rpm for 10 min at 4°C, then washed with 3 ml cold PBS, pelleted again, and finally suspended in 200 μl of 0.9% NaCl, 0.01% EDTA, pH 7.4. The cell suspension was sonicated on ice and an aliquot was immediately removed and added to a pre-chilled tube containing 4 M guanidine isothiocyanate. Total cellular RNA was purified from the cell extract using previously published procedures (32). Purified RNA was dissolved in diethyl pyrocarbonate-treated water and the concentration was determined by the absorbance at 260 nm. Integrity of the purified RNA was determined by formaldehyde agarose gel electrophoresis as previously described (33). Cellular apoA-I, B, E, and LDL receptor mRNA abundance were quantified using DNA-excess solution hybridization assay as described previously (32, 33).

Apolipoprotein measurement by ELISA

Cell medium was removed at the appropriate times and centrifuged at 2,000 rpm for 5 min to remove cell debris. One-ml aliquots were taken and placed in 0.01% BSA-coated tubes and stored at −100°C until quantification. Apolipoprotein A-I, B, and E accumulation in culture medium was quantified by ELISA as previously described (34). Aliquots of the sonicated cell lysate were saved for protein quantification (35). These values were normalized to the total cellular protein content per dish and expressed as μg apolipoprotein/mg cell protein.

Isolation and iodination of LDL

Human LDL were isolated by density gradient centrifugation and iodinated using the previously described iodine monochloride procedure (36). Radiolabeled LDL was dialyzed for 5 days with five changes of PBS containing 0.01% EDTA and 0.1 M NaI to remove free iodide. Greater than 99% of the labeled LDL was found to be trichloroacetic acid-precipitable.

Quantification of LDL binding, internalization, and degradation

Cells were grown on 35-mm dishes in MEM + 10% FBS to 85–90% confluence (approximately $8.7 \cdot 10^5$ cells/dish). The monolayer was washed and the medium was changed to MEM containing 75 μM BSA and incubated for 24 h. The cell monolayer was gently washed twice with PBS and then incubated with FBS-free MEM supplemented with one of various additions: 75 μM BSA; 150 μM 22:6 complexed to 75 μM BSA; 75 μM BSA and 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol; or 150 μM 22:6 complexed to 75 μM BSA and 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol. After a 6 h incubation at 37°C, the ^{125}I-labeled LDL was added to the appropriate medium and the cells were incubated for an additional 4 h, after which they were maintained at 4°C. LDL receptor binding, degradation, and internalization were measured using the procedures described by Brown and Goldstein (37). Since the results from LDL binding, internalization, and degradation studies showed similar differences among treatment groups, only LDL degradation results are shown.

Uptake of secreted HepG2 cell apoB-containing lipoproteins

Six T-150 flasks of HepG2 cells were grown to 85–90% confluence on MEM + 10% FBS. The monolayers were washed with PBS and incubated in FBS-free MEM supplemented with 150 μM 22:6 complexed to 75 μM BSA and 50 μg/ml cholesterol + 10 μg/ml 25-hydroxycholesterol containing 300 μCi of L-[3, 4, 5-^{3}H(N)]leucine in 20 ml of medium and incubated for 6 h. The combined HepG2 cell culture medium was harvested in 0.5% ethanolamine-treated polypropylene tubes and the density was adjusted to d 1.080 g/ml with KBr. This mixture was overlayered with a KBr solution at d 1.063 g/ml and ultracentrifuged in a SW 40 rotor for 40 h at 36,000 rpm. The d < 1.063 g/ml fraction was removed and dialyzed extensively against PBS to remove traces of labeled free leucine. The ^{3}H-labeled apoB-containing lipoproteins were further concentrated approximately 2-fold by the use of dextran T-500, and were then dialyzed against MEM. Greater than 99% of the ^{3}H-labeled apoB-containing lipoproteins were found to be trichloroacetic acid-precipitable.

HepG2 cells grown to 85–90% confluence on 35-mm dishes were treated with FBS-free MEM containing 75 μM BSA for 24 h, after which they were switched to MEM containing either 75 μM BSA or 75 μM BSA and 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol for 6 h. Immediately after this treatment, cells were incubated with MEM supplemented with either BSA or BSA + 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol, and an aliquot of the ^{3}H-labeled apoB-containing lipoproteins (180,807 dpm/dish) for 18 h at 37°C. Culture medium was then removed and the radioactivity was measured. The cell monolayer was extensively washed (37) to remove nonspecifically bound labeled lipoproteins. Internalized ^{3}H-labeled apoB-containing lipoproteins were isolated by digestion of the cell monolayer with NaOH (37) and quantified by liquid scintillation counting. Internalized ^{3}H-labeled apoB-containing lipoproteins were expressed as dpm/dish and as the fraction of the total added ^{3}H-labeled apoB-containing lipoproteins internalized.

Data analysis

Values are given as the mean ± standard error of the mean. Statistical comparisons were made using the Student's *t* test.

RESULTS

Apolipoprotein accumulation time-course

Apolipoprotein (apo) A-I, B and E accumulation in culture medium was measured after 0, 3, 6, 12, and 24 h of incubation with 150 μM docosahexaenoic acid complexed to 75 μM BSA (BSA 22:6), either in the presence or absence of 50 μg/ml cholesterol and 4 μg/ml 25-hydroxycholesterol (C/25-OH). **Fig. 1**, panel A, shows that the apoA-I concentration in the medium increased with time but with no significant differences among treatment groups. In contrast, apoB accumulation (Fig. 1, panel B) showed significant differences among all four treatment groups. Most striking was the lack of further apoB accumulation after 6 h from cells treated with BSA only. ApoB accumulation was 1.7-fold greater in the presence of BSA + C/25-OH after 24 h, and 2.0-fold greater in the presence of BSA-22:6, when compared to BSA treatment alone. The combination of BSA-22:6 + C/25-OH for 24 h increased apoB accumulation approximately 5.0-fold as compared to BSA treatment alone. ApoE accumulation increased with time and was significantly greater in the presence of BSA + C/25-OH as shown in Fig. 1, panel C. The accumulation of apoE in the presence of BSA + C/25-OH for 24 h was approximately 1.8-fold greater than for cells grown in the absence of C/25-OH.

ApoA-I accumulated linearly in all treatment groups, 3.25 ± 0.2 μg/mg cell protein per 24 h. ApoE accumulation was linear in all treatment groups but was significantly higher ($P < 0.001$) in cells incubated with C/25-OH, averaging, 0.19 ± 0.009 μg/mg cell protein per 24 h; in the absence of C/25-OH, the average was 0.10 ± 0.02 μg/mg cell protein per 24 h. These differences in apoE accumulation, as for apoB, were seen between 12 and 18 h of incubation. However, HepG2 cell apoB, unlike apoE accumulation, was also affected by fatty acid treatment, averaging 1.1 ± 0.05 μg/mg cell protein per 24 h for BSA alone; 1.95 ± 0.1 μg/mg cell protein per 24 h for BSA + C/25-OH; 2.6 ± 0.15 μg/mg cell protein per 24 h for BSA-22:6; and 5.5 ± 0.25 μg/mg cell protein per 24 h for BSA-22:6 + C/25-OH. These differences in apoB accumulation were seen between 12 and 18 h with respect to both BSA-22:6 and C/25-OH treatments.

Comparative studies using BSA-18:1 treatment were carried out for 24 h under identical experimental conditions and showed 3.09 ± 0.35, 3.11 ± 0.25, and 0.11 ± 0.02 μg/mg cell protein per 24 h (mean ± SEM, n = 4) for apoA-I, B, and E accumulation, respectively. The apoB accumulation for BSA-22:6 treatment (2.60 ± 0.15 μg/mg cell protein per 24 h) was compared to BSA-18:1 treatment (3.11 ± 0.25 μg/mg cell protein per 24 h) and found not to be statistically different at the $P < 0.05$ level.

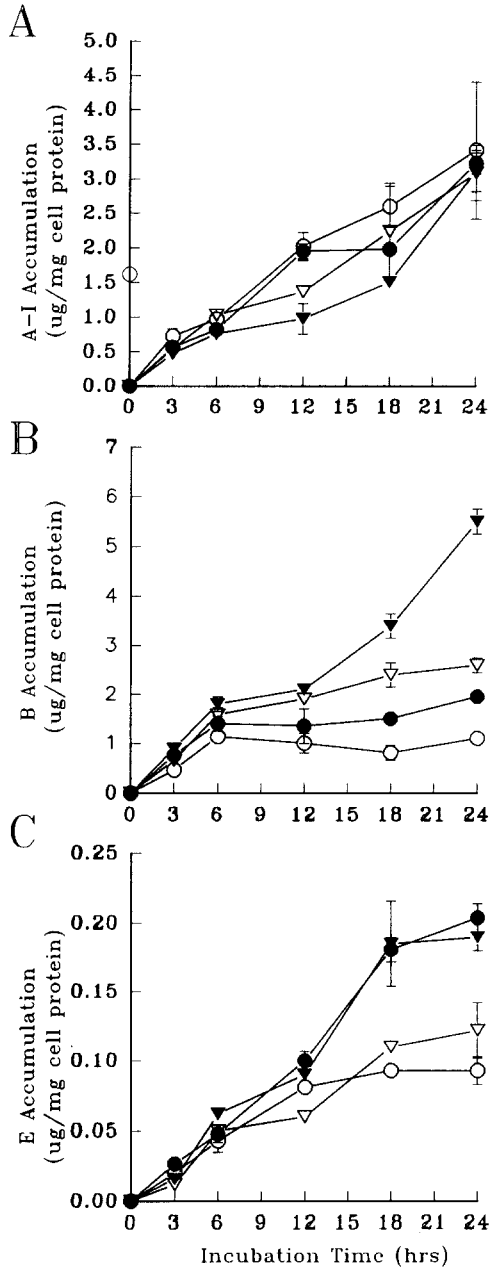


Fig. 1. Apolipoprotein accumulation from HepG2 cells expressed as μg/mg cell protein after incubation with 75 μM fatty acid-free bovine serum albumin (BSA) (○), 150 μM docosahexaenoic acid complexed to 75 μM BSA (BSA-22:6) (▽), or these albumin preparations in the presence of 50 μg/ml cholesterol and 4 μg/ml 25-hydroxycholesterol (C/25-OH) (●) and (▼), respectively. HepG2 cells grown in 60-mm dishes were pretreated for 24 h in MEM containing 75 μM BSA and then switched to one of the four treatments and incubated for 3, 6, 12, 18, and 24 h. At the appropriate times, cells and culture media were harvested. Panel A, apoA-I; panel B, apoB; and panel C, apoE. Concentrations were determined by ELISA and normalized to total cellular protein as described in Materials and Methods. These values represent the mean ± SEM of triplicate dishes of cells from at least three independent experiments.

TABLE 1. HepG2 cell triglycerides after 24 h treatment with BSA or BSA-complexed fatty acids

Treatment	Triglyceride
	nmol/mg cell protein
BSA	119 ± 34[a,b]
18:1	186 ± 22[c]
22:6	244 ± 31

Values represent the mean ± SD of a single experiment carried out using triplicate 100-mm plates of cells for each treatment.
[a]BSA versus 18:1, $P < 0.01$.
[b]BSA versus 22:6, $P < 0.02$.
[c]18:1 versus 22:6, $P < 0.05$.

Fatty acid stimulation of HepG2 cell triglyceride mass was determined after treatment of cells for 24 h. After fatty acid treatment, HepG2 cell pellets were harvested and their triglyceride mass content was determined. **Table 1** shows that cellular triglyceride accumulation was stimulated by both BSA-22:6 and -18:1 treatment, as compared to BSA treatment. HepG2 cells accumulated more cellular triglyceride with BSA-22:6 treatment (244 nmol/mg cell protein), as compared to BSA-18:1-treated cells (186 nmol/mg cell protein), which is consistent with results from other investigators (12).

Effects of fatty acid and cholesterol on apolipoprotein mRNA abundance

The abundance of apoA-I, B, and E mRNA in HepG2 cells was quantified after treatment with 150 μM 22:6 complexed to 75 μM BSA, either in the presence or absence of C/25-OH. At the appropriate times the total cellular RNA was isolated and used for analysis of mRNA abundance. **Fig. 2** shows HepG2 cell mRNA abundance for apoA-I, (panel A), apoB, (panel B), and apoE, (panel C) for each of the four treatments. ApoA-I, B, and E mRNA abundance was found to be unaffected by dietary treatments or length of incubation; over the course of the study the average values were 48.2 ± 7.5, 222 ± 33.6, and 17.1 ± 0.7 pg mRNA/μg RNA (mean ± SEM), respectively.

Regulation of LDL receptor mRNA and receptor activity

To assess the involvement of HepG2 cell LDL receptor expression on the accumulation of apoB and apoE with time, LDL receptor mRNA and receptor activity were also quantified over a 24-h time course. BSA + C/25-OH treatment reduced HepG2 cell LDL receptor mRNA approximately 2.3-fold as compared to BSA-treated cells, as shown in **Fig. 3**, panel A. LDL receptor mRNA expressed as pg mRNA/ μg RNA averaged 7.1 ± 0.3 for BSA, 2.6 ± 0.1 for BSA + C/25-OH, 5.5 ± 0.2 for BSA-22:6, and 2.3 ± 0.2 for BSA-22:6 + C/25-OH (mean ± SEM) by 6 h of incubation. The decrease in LDL receptor mRNA abundance was apparent by 6 h and persisted for 18 h. Beyond 18 h of incubation it appeared that HepG2 cell LDL receptor mRNA levels had begun to increase to their pre-treatment levels, presumably due to depletion of intracellular levels of C/25-OH. No significant differences were seen in LDL receptor mRNA abundance between cells treated with BSA-22:6 as compared to BSA treatment alone.

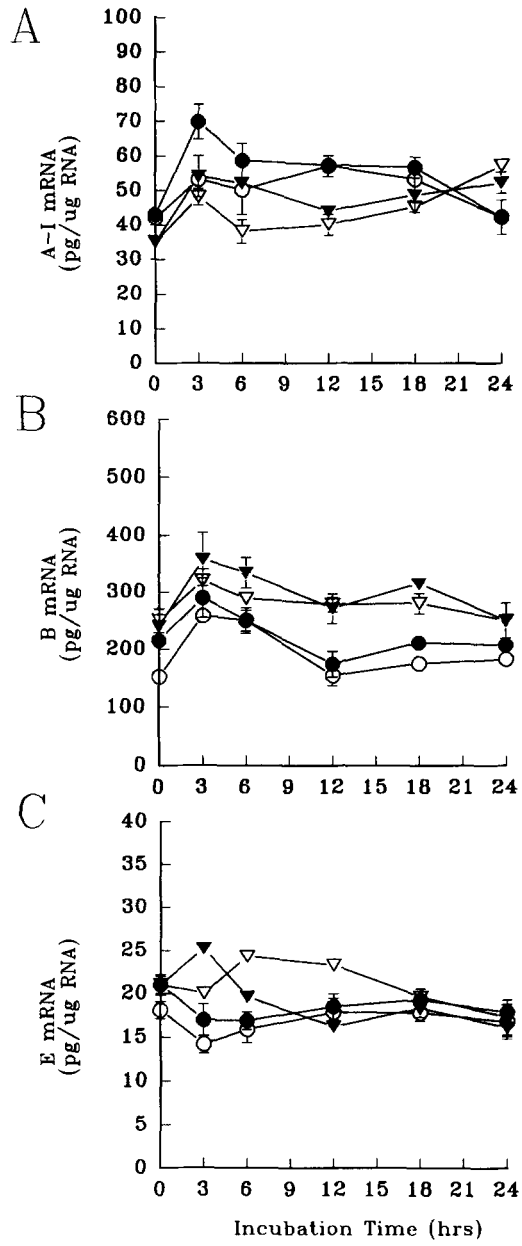
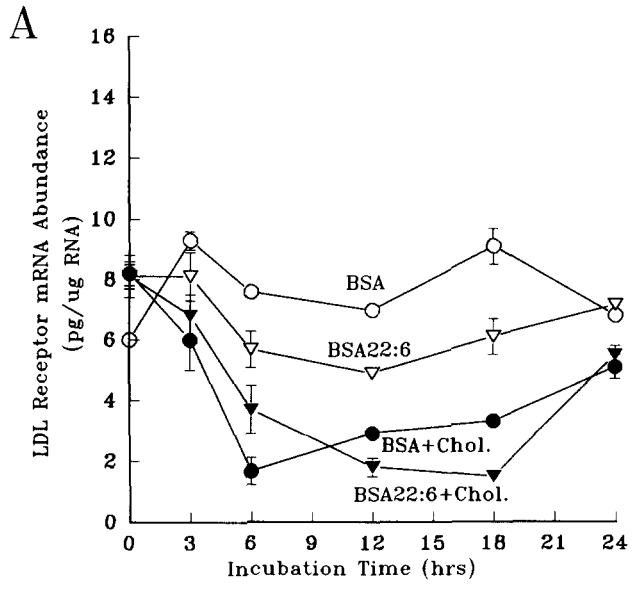


Fig. 2. Apolipoprotein mRNA abundance in HepG2 cells expressed as pg mRNA/μg RNA after incubation with one of the four treatments as described in the legend to Fig. 1. BSA (○); BSA-22:6 (▽); BSA + C/25-OH (●); BSA-22:6 + C/25-OH (▼). Total cellular RNA was isolated and the mRNA abundance was measured as described in Materials and Methods. Panel A, apoA-I, panel B, apoB; and panel C, apoE. These values represent the mean ± SEM of triplicate dishes of cells from at least three independent experiments.



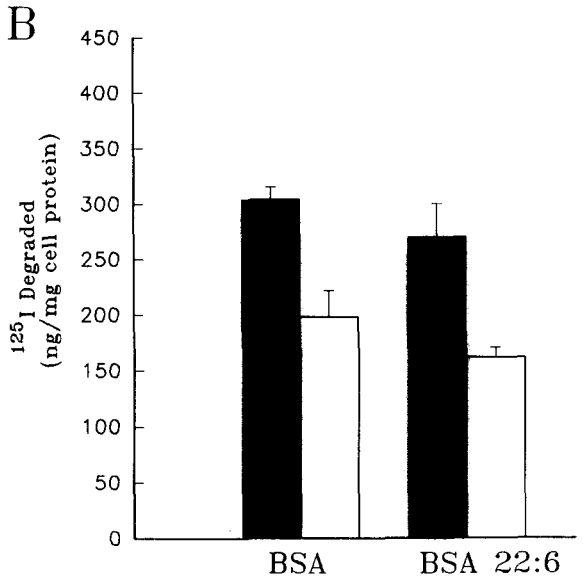


Fig. 3. LDL receptor mRNA abundance in HepG2 cells, panel A, expressed as pg mRNA/ μg RNA after incubation with one of the four treatments as described in the Fig. 1 legend. These values represent the mean ± SEM of triplicate dishes of cells from at least three independent experiments. Panel B, LDL receptor activity expressed as ng ^{125}I-labeled LDL degraded /mg cell protein was determined as described in Materials and Methods. These values represent the mean ± SEM of triplicate dishes of cells from at least three independent experiments. Cells were treated for 6 h with 75 μM fatty acid-free bovine serum albumin (BSA) or 150 μM docosahexaenoic acid complexed to 75 μM BSA (BSA-22:6) either in the presence (open bars), or absence (solid bars) of 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol.

LDL receptor activity was measured after 6 h of treatment of HepG2 cells with each of the four conditions. Degradation of ^{125}I-labeled LDL was increased approximately 1.5-fold in cells treated with BSA alone, as compared to BSA + C/25-OH as shown in Fig. 3, panel B. Internalized LDL expressed as ng ^{125}I-labeled LDL/ mg protein averaged 310.4 ± 12.2 for BSA, 167.4 ± 8.9 for BSA + C/25-OH, 280.6 ± 11.4 for BSA-22:6, and 158.6 ± 5.5 for BSA-22:6 + C/25-OH (mean ± SEM). No significant differences in LDL receptor activity were seen between BSA-22:6-and BSA only-treated cells.

Uptake of secreted HepG2 apoB-containing lipoproteins

To determine the extent to which HepG2 cell-derived apoB-containing lipoproteins could be removed by their own receptor-mediated pathways, labeled apoB-containing lipoproteins were prepared by incubating HepG2 cells with [^{3}H]leucine. The labeled medium was ultracentrifuged after incubation and the d < 1.063 g/ml fraction was isolated and dialyzed to obtain ^{3}H-labeled apoB-containing lipoproteins. HepG2 cells pre-treated with BSA or BSA + C/25-OH for 6 h were incubated with fresh medium supplemented with ^{3}H-labeled apoB-containing lipoproteins (180,807 dpm/dish) for 18 h at 37°C. After this period, the medium was removed and the cells were washed extensively to remove nonspecifically bound lipoproteins. Cells treated with BSA showed 44.7% (80,916 ± 2,597 dpm) of the ^{3}H-labeled apoB-containing lipoproteins had been internalized, while cells treated with BSA + C/25-OH showed 36.0% (65,338 ± 3,826 dpm) had been internalized, as shown in **Fig. 4**. This difference in the amount of ^{3}H-labeled apoB-containing lipoproteins internalized was found to be significant at $P < 0.004$. Cells treated with BSA showed 62.5% (113,054 ± 2,930 dpm) of the ^{3}H-labeled lipoproteins remained in the medium as compared to 70.2%

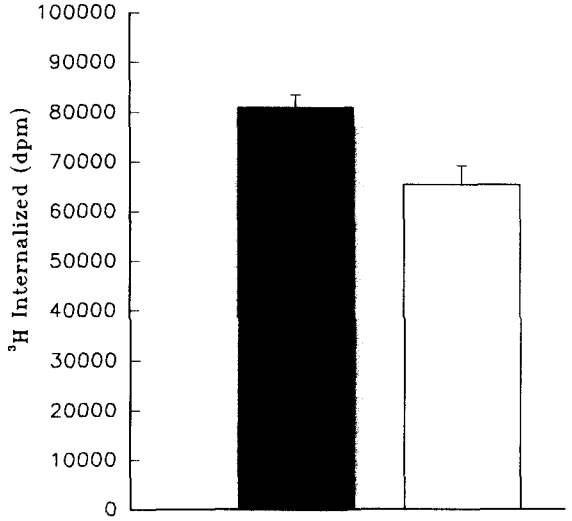


Fig. 4. LDL receptor removal of HepG2 cell-derived ^{3}H-labeled apoB-containing lipoproteins. HepG2 cells were treated with MEM containing 180,807 dpm/dish of [^{3}H]leucine-labeled apoB-containing lipoproteins and either 75 μM BSA + 50 μg/ml cholesterol + 4 μg/ml 25-hydroxycholesterol (open bar), or 75 μM BSA (solid bar). These values represent the mean ± SEM of triplicate dishes of cells.

(127,073 ± 6,888) for cells treated with BSA + C/25-OH. This difference was found to be significant at $P < 0.03$. These results show an approximate 1.25-fold higher uptake of ^{3}H-labeled apoB-containing lipoproteins resulted when incubated with HepG2 cells treated with BSA as compared to cells treated with BSA + C/25-OH.

DISCUSSION

The present study was undertaken to establish the relationship between HepG2 cell apolipoprotein gene expression and LDL receptor activity, and the degree to which LDL receptor activity influences apolipoprotein accumulation. These studies show that by 12 h of BSA + C/25-OH treatment, accumulation of both apoB and apoE was stimulated approximately 2.0-fold as compared to cells treated only with BSA. When BSA-22:6 and C/25-OH were both included in the incubation, apoB accumulation was stimulated 5.0-fold as compared to BSA-treated cells, while apoE accumulation was not stimulated by BSA-22:6 treatment. Furthermore, the increase in apoB and E mass accumulation in response to C/25-OH treatment was correlated with the down-regulation of HepG2 cell LDL receptor activity. A similar stimulation of apoB and E in HepG2 cells has been shown by Craig, Nutik, and Cooper (17) using a somewhat different approach. In these studies a 7- to 14-fold increase in apoB and E accumulation was found upon the preincubation of HepG2 cells with either 40 μg/ml of βVLDL or chylomicron remnants, respectively. These investigators hypothesized that an increased cholesterol supply is necessary for stimulation of synthesis and secretion of apoB-containing lipoproteins (both VLDL and LDL) as incubation of HepG2 cells with chylomicron remnants increased apolipoprotein secretion but did not alter the number of LDL receptors expressed (17). Other investigators (25, 38) have also demonstrated that cholesterol treatment stimulates apoB accumulation in HepG2 cell culture medium. It is therefore a possibility that in our studies the addition of cholesterol and 25-hydroxycholesterol increases apoB secretion, as well as decreasing LDL receptor activity. In one study (25) the rates of apoB secretion and LDL receptor activity were negatively correlated; however, these investigators did not find any significant interaction of HepG2 cell-derived apoB-containing particles with cells and concluded that apoB accumulation in the medium does provide an acceptable indication of the synthetic rates.

Although expression of LDL receptors in HepG2 cells has been demonstrated previously (25–30), only recently have HepG2 cells been shown to be capable of removing HepG2 cell-derived lipoproteins (20, 26, 27). Williams, Brocia, and Fisher (26) have proposed that apoB reuptake by HepG2 cells is predominately local. They hypothesize that local re-uptake from the unstirred water layer may be a general mechanism for secretory control. In our studies, we have determined the magnitude to which HepG2 cell LDL receptor activity can alter the overall mass accumulation of apoB and E in culture medium as a function of time. In our system, HepG2 cells removed 1.25-fold less HepG2 cell-derived ^{3}H-labeled apoB-containing lipoproteins when pre-treated with BSA + C/25-OH than BSA controls. The extent to which HepG2 cells can remove newly secreted apoB-containing lipoproteins appears to be slightly less than the uptake of plasma-derived LDL particles, but the re-uptake process does explain a proportion of the 2-fold difference in accumulation seen between C/25-OH- and BSA-treated cells. Therefore, it appears that uptake of newly synthesized HepG2 cell lipoproteins contributes to the apparently lower accumulation of apoB and E from cells incubated in the absence of C/25-OH. The difference in the degree of removal of ^{125}I-labeled LDL versus ^{3}H-labeled apoB-containing lipoprotein by HepG2 cells may be a result of differences in the composition and apolipoprotein content of the respectively labeled lipoproteins used in these studies. However, it is also possible that hepatic lipase activity, which has been measured in our HepG2 cell culture medium (39) after heparin treatment (M. Sorci-Thomas and T. Thuren, unpublished results), converts only a portion of the nascent HepG2 cell apoB-containing lipoproteins into an LDL receptor-competent substrate.

In these studies only HepG2 cell apoB accumulation increased in response to BSA-22:6, while apoA-I and E accumulation levels were unchanged by this treatment. These data agree with results from several other laboratories that show that BSA-complexed fatty acids increase apoB accumulation in HepG2 cell culture medium (13, 18–23), but differ from those reports that show small or nonexistent stimulation of apoB accumulation with fatty acid treatment (12, 14–17). In particular, Wong and Nestel (13) have shown that treatment of HepG2 cells for 6 h with 1 mM 18:1 or 18:2 increased apoB secretion 2-fold relative to BSA, while 20:5 modestly decreased (16%) VLDL apoB secretion, compared to BSA control. In another study (12), cells treated for 3 h with 1 mM 20:5 or 22:6 secreted 1.4- and 2.2-fold less [^{3}H]leucine-labeled VLDL apoB, respectively, in the culture medium as compared to either 18:1- or BSA only-treated cells, in which nearly equivalent levels of accumulation were observed. The reasons for the differences in apoB accumulation in response to 18:1 and 22:6 treatment between this study (12) and the present study are not apparent. In our study, HepG2 cells were pre-treated with BSA for 24 h (18) to achieve a common fatty acid-depleted baseline from which to observe the effects of BSA-complexed fatty acids on apolipoprotein gene expression. Other factors related to differences in the cell culture conditions, cell number, or confluency may also be responsible. In our studies, like those of Wong and Nestel (13), we showed increased cellular triglyceride ac-

cumulation with both 18:1- and 22:6-treated cells, with a significantly higher accumulation of triglyceride mass observed in cells treated with 22:6 as compared to 18:1. Therefore, similarities in HepG2 cellular triglyceride response between the two studies are apparent.

It has been documented by several investigators that 18:1 has a differential effect in HepG2 cells as compared to primary rat hepatocytes. In rat hepatocytes, 18:1 does not stimulate apoB secretion compared to BSA treatment alone (8–10), while in this study and in many others, (13, 18–23), 18:1 does stimulate apoB secretion from HepG2 cells. On the other hand, in rat hepatocytes (8–10), as in HepG2 cells (12–19, 40), triglyceride secretion is stimulated by 18:1 treatment. One explanation for the observed difference between HepG2 cell and rat hepatocyte apoB expression in response to 18:1 (and 22:6 as demonstrated in our studies) was suggested by Dixon, Furukawa, and Ginsberg (20). As HepG2 cells grow rapidly and require large amounts of energy, they may resemble a "fasted" hepatocyte more than a "fed" hepatocyte. According to the model, this deficient metabolic state may explain why fatty acid treatment stimulates apoB-containing lipoprotein secretion from HepG2 cells. Similarly, Salam, Wilcox, and Heimberg (7) have shown that 18:1 stimulates apoB secretion in perfused livers from 24-h-fasted rats, but not in livers from fed rats.

Over the 24-h time course studied, we have demonstrated that fatty acid and cholesterol treatment does enhance apoB and E accumulation but has little or no effect on apoA-I or E mRNA abundance. Previous reports have demonstrated an increase in apoE, and A-I (28, 41) mRNA and apolipoprotein accumulation in response to BSA + cholesterol or 25-hydroxycholesterol treatment using HepG2 cells. The present studies also show that apoB and E accumulation increased when cells were treated with BSA 22:6 + cholesterol/25-hydroxycholesterol as compared to BSA-22:6-treated cells. However, in our studies, increased apoB and E accumulation was found to be independent of changes in apoB or apoE mRNA abundance. Other investigators have previously shown that HepG2 cell apoA-I, B, and E mRNA abundance levels were unaffected by oleate (18:1) (12, 16, 18, 19), and HepG2 cell apoB mRNA levels were unaffected by eicosapentaenoic acid (20:5) or docosahexaenoic acid (22:6) treatment (12). The lack of fatty acid modification on apolipoprotein mRNA abundance, as shown in these studies, also agrees with studies carried out in nonhuman primates (33), except for the results with apoA-I mRNA (42). Studies in African green monkeys fed polyunsaturated fat and cholesterol show a 25% lower liver apoA-I mRNA abundance as compared to lard-fed animals, while African green monkeys fed diets containing fish oil and cholesterol (5) show an approximately 33% lower liver apoA-I mRNA relative to their lard-fed counterparts (M. Sorci-Thomas, J. Parks, and L. Rudel, unpublished results). Recently, studies carried out in cebus monkeys (43) showed a dietary corn oil-related decrease in hepatic apoA-I mRNA abundance when compared to results from coconut oil-fed animals. Overall, these results suggest that modulation of HepG2 cell apoA-I mRNA by fatty acid and cholesterol treatment may require more extensive treatment times or dosages than were used in this study. It is also possible that the dietary modulation of apoA-I mRNA abundance demonstrated using whole animals cannot be modeled in the human-derived HepG2 cell line.

In summary, we postulate that HepG2 cell LDL receptor activity mediates the removal of apoB, E-containing lipoproteins from culture medium and contributes to the lower accumulation of apoB and E observed in culture medium from cells treated with BSA as compared to cells treated with C/25-OH.

We thank Dr. Trudy Forte for supplying the HepG2 cells and Lynda Farley for excellent technical assistance. We also thank Drs. Michael Thomas, John Parks, and Greg Shelness for critical discussions of this manuscript. This work was supported by NIH grants from the National Heart, Lung, and Blood Institute, HL-41916 and HL-14164.

Manuscript received 3 October 1991, in revised form 24 February 1992, and in re-revised form 1 April 1992.

REFERENCES

1. Bang, H. O., and J. Dyerberg. 1972. Plasma lipids and lipoproteins in Greenlandic west coast Eskimos. *Acta Med. Scand.* **192:** 85–94.
2. Kromhout, D., E. B. Bosschieter, and C. de Lezenne Coulander. 1985. The inverse relation between fish consumption and 20 year mortality from coronary heart disease. *N. Engl. J. Med.* **312:** 1205–1209.
3. Harris, W. S., W. E. Connor, and M. P. McMurry. 1983. The comparative reductions of the plasma lipids and lipoproteins by dietary polyunsaturated fats:salmon oil vs. vegetable oils. *Metabolism.* **32:** 179–184.
4. Harris, W. S. 1989. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *J. Lipid Res.* **30:** 785–807.
5. Parks, J. S., and L. L. Rudel. 1990. Effects of fish oil on atherosclerosis and lipoprotein metabolism. *Atherosclerosis.* **84:** 83–94.
6. Parks, J. S., J. Kaduck-Sawyer, B. C. Bullock, and L. L. Rudel. 1990. Effects of dietary fish oil on coronary artery and aortic atherosclerosis in African green monkeys. *Arteriosclerosis.* **10:** 1102–1112.
7. Salam, W. H., H. G. Wilcox, and M. Heimberg. 1988. Effects of oleic acid on the biosynthesis of lipoprotein apoproteins and distribution into the very-low density lipoprotein by the isolated perfused rat liver. *Biochem. J.* **251:** 809–816.
8. Davis, R. A., S. C. Engelhorn, S. H. Pangburn, D. B. Weinstein, and D. Steinberg. 1979. Very low density lipoprotein synthesis and secretion by cultured rat hepatocytes. *J. Biol. Chem.* **254:** 2010–2016.

9. Wong, S. H., M. Reardon, and P. J. Nestel. 1985. Reduced triglyceride formation from long-chain polyenoic fatty acids in rat hepatocytes. *Metabolism.* **34:** 900–904.
10. Lang, C. A., and R. A. Davis. 1990. Fish oil fatty acids impair VLDL assembly and/or secretion by cultured rat hepatocytes. *J. Lipid Res.* **31:** 2079–2086.
11. Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, HepG2, under defined conditions. *J. Lipid Res.* **27:** 236–250.
12. Wong, S-H., E. A. Fisher, and J. B. Marsh. 1989. Effects of eicosapentaenoic and docosahexaenoic acids on apoprotein B mRNA and secretion of very low density lipoprotein in HepG2 cells. *Arteriosclerosis.* **9:** 836–841.
13. Wong, S., and P. J. Nestel. 1987. Eicosapentaenoic acid inhibits the secretion of triacylglycerol and of apoprotein B and the binding of LDL in HepG2 cells. *Atherosclerosis.* **64:** 139–146.
14. Dashti, N., and G. Wolfbauer. 1987. Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: effects of oleic acid and insulin. *J. Lipid Res.* **28:** 423–436.
15. Ellsworth, J. L., S. K. Erickson, and A. D. Cooper. 1986. Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J. Lipid Res.* **27:** 858–874.
16. Dashti, N., D. L. Williams, and P. Alaupovic. 1989. Effects of oleate and insulin on the production rates and cellular mRNA concentration of apolipoproteins in HepG2 cells. *J. Lipid Res.* **30:** 1365–1373.
17. Craig, W. Y., R. Nutik, and A. D. Cooper. 1988. Regulation of apoprotein synthesis and secretion in the human hepatoma HepG2. *J. Biol. Chem.* **263:** 13880–13890.
18. Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1077.
19. Moberly, J. A., T. G. Cole, D. H. Alpers, and G. Schonfeld. 1990. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. *Biochim. Biophys. Acta.* **1042:** 70–80.
20. 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.* **256:** 5080–5086.
21. Rash, J. M., G. H. Rothblat, and C. E. Sparks. 1981. Lipoprotein apolipoprotein synthesis by human hepatoma cells in culture. *Biochim. Biophys. Acta.* **666:** 294–298.
22. Boström, D., J. Borén, M. Wettesten, A. Sjöberg, G. Bondjers, O. Wiklund, P. Carlsson, and S-O. Olofsson. 1988. Studies on the assembly of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **263:** 4434–4442.
23. Erickson, S. K., and P. E. Fielding. 1986. Parameters of cholesterol metabolism in the human hepatoma cell line, Hep-G2. *J. Lipid Res.* **27:** 875–883.
24. Davis, R. A., and J. R. Boogaerts. 1982. Intrahepatic assembly of very low density lipoproteins. Effects of fatty acids on triacylglycerol and apolipoprotein synthesis. *J. Biol. Chem.* **257:** 10908–10913.
25. Fuki, I. V., S. N. Preobrazhensky, A. Y. Misharin, N. G. Bushmakina, G. B. Menschikov, G. B., V. S. Repin, and R. S. Karpov. 1989. Effects of cell cholesterol content on apolipoprotein B secretion and LDL receptor activity in the human hepatoma cell line, HepG2. *Biochim. Biophys. Acta.* **1001:** 235–238.
26. Williams, K. J., R. W. Brocia, and E. A. Fisher. 1991. The unstirred water layer as a site of control of apolipoprotein B secretion. *J. Biol. Chem.* **265:** 16741–16744.
27. Hara, S., M. McCall, and T. Forte. 1990. Re-uptake of nascent low density lipoproteins (LDL) by HepG2 cells. *FASEB J.* **4:** A656.
28. Carlson, T. L., and B. A. Kottke. 1989. Effects of 25-hydroxycholesterol and bile acids on the regulation of cholesterol metabolism in HepG2 cells. *Biochem. J.* **264:** 241–247.
29. Ranganathan, S., and B. A. Kottke. 1989. Studies on the regulation of cholesterol metabolism by low- and high-density lipoproteins in HepG2 cells. *Hepatology.* **9:** 547–551.
30. Havekes, L., V. Van Hinsbergh, H. J. Kempen, J. Emeis. 1983. The metabolism in vitro of human low-density lipoproteins by the human hepatoma cell line HepG2. *Biochem. J.* **214:** 951–958.
31. Van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. *J. Biol. Chem.* **244:** 2278–2285.
32. Sorci-Thomas M., M. M. Prack, N. Dashti, F. Johnson, L. L. Rudel, and D. L. Williams. 1988. Apolipoprotein (apo) A-I production and mRNA abundance explain plasma apoA-I and high density lipoprotein differences between two nonhuman primate species with high and low susceptibilities to diet-induced hypercholesterolemia. *J. Biol. Chem.* **263:** 5183–5189.
33. Sorci-Thomas, M., M. Wilson, F. L. Johnson, D. L. Williams, and L. L. Rudel. 1989. Studies on the expression of genes encoding apolipoproteins B-100 and B-48 and the LDL receptor in nonhuman primates. Comparison of dietary fat and cholesterol. *J. Biol. Chem.* **264:** 9039–9045.
34. Koritnik, D. L., and L. L. Rudel. 1983. Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). *J. Lipid Res.* **24:** 1639–1645.
35. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
36. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature.* **182:** 53–57.
37. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1977. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–261.
38. Dashti, N. 1990. Regulation of apolipoprotein B gene expression by cholesterol in HepG2 cells. *Circulation.* **82 (suppl III):** 2106.
39. Busch, S. J., G. A. Martin, R. L. Barnhart, and R. L. Jackson. 1989. Heparin induces the expression of hepatic triglyceride lipase in human hepatoma (HepG2) cell line. *J. Biol. Chem.* **264:** 9527–9532.
40. Homan, R., J. E. Grossman, and H. J. Pownall. 1991. Differential effects of eicosapentaenoic acid and oleic acid on lipid synthesis and secretion by HepG2 cells. *J. Lipid Res.* **32:** 231–241.

41. Monge, J. C., J. M. Hoeg, S. W. Law, and H. B. Brewer, Jr. 1989. Effects of low density lipoproteins, high density lipoproteins, and cholesterol on apolipoprotein A-I mRNA in HepG2 cells. *FEBS Lett.* **243:** 213–217.
42. Sorci-Thomas, M., M. M. Prack, N. Dashti, F. L. Johnson, L. L. Rudel, and D. L. Williams. 1989. Differential effects of dietary fat on the tissue-specific expression of the apolipoprotein A-I gene: relationship to plasma concentration of high density lipoproteins. *J. Lipid Res.* **30:** 1397–1403.
43. Stucchi, A., L. K. Hennessy, D. B. Vespa, E. J. Weiner, J. Osada, J. M. Ordovas, E. J. Schaefer, and R. J. Nicolosi. 1991. Effect of corn and coconut-oil containing diets with and without cholesterol on HDL apoA-I metabolism and hepatic apoA-I mRNA levels in cebus monkeys. *Arteriosclerosis.* **11:** 1719–1729.