Effect of ethanol on the synthesis and secretion of apoA-I- and apoB-containing lipoproteins in HepG2 cells

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Abstract The short- and long-term effects of ethanol on the production of lipids and apolipoproteins (apo) in HepG2 cells were studied. Short-term incubation with 1% ethanol caused a significant 32% increase in the cellular content of both triglycerides and cholesteryl esters. Under these conditions, the net accumulation in the medium of triglycerides, unesterified cholesterol, cholesteryl esters, apoA-I, and apoE was stimulated by 75%, 41%, 43%, 19%, and 39%, respectively. ApoA-I and apoE mRNA levels increased by 15%. The major short-term effect of ethanol was on the net accumulation of apoB in the medium which was stimulated by 56–100% in the presence of 0.1–1.0% ethanol. Under these conditions, apoB mRNA abundance was elevated by 17–26% and LDL receptor activity was unchanged. The increase in apoB accumulation in the medium was predominantly due to augmented secretion of newly synthesized apoB-100 which was evident at 0.05% ethanol. The secretion of newly synthesized apoA-I was not altered by short-term incubation with ≤ 0.5% ethanol. The rate of apoB production was positively correlated with the cellular and secreted cholesteryl esters and secreted triglycerides. Addition of Pfizer CP-113,818, an inhibitor of acyl-CoA:cholesterol acyltransferase, caused a 69% reduction in the secretion of cholesteryl esters and a 24% decrease in that of apoB-100. In contrast to the short-term effect of ethanol, long-term incubation with ethanol resulted in a dose-dependent increase in the secretion of newly synthesized apoA-I without significantly affecting that of apoB-100. The increase in apoA-I secretion was evident at 0.05% ethanol and reached a maximum of 77% at 0.5% ethanol. These observations indicate that in HepG2 cells the effect of ethanol on the production of apoA-I- and apoB-containing lipoproteins is both time- and dose-dependent and is different in these two apolipoproteins.—**Dashti, N., F. A. Franklin, and D. R. Abrahamson.** Effect of ethanol on the synthesis and secretion of apoA-I- and apoB-containing lipoproteins in HepG2 cells. *J. Lipid Res.* 1996. **37:** 810–824.

Supplementary key words triglycerides • cholesteryl esters • unesterified cholesterol • apoE • liver • mRNA • LDL receptor • acyl-CoA:cholesterol acyltransferase • Pfizer CP-113,818

The positive correlation between the serum level of low density lipoproteins (LDL) and the incidence of coronary artery disease (1, 2), and the negative association between high density lipoprotein (HDL) cholesterol and the development of atherosclerosis (3, 4) are well established. In addition to plasma LDL- and HDL-cholesterol, low concentration of apoA-I and increased level of apoB have been implicated as positive risk factors for cardiovascular disease (5). Numerous studies have consistently demonstrated that moderate alcohol intake exerts a protective effect against coronary heart disease (6–11). By contrast, consumption of excessive amounts of alcohol may actually increase the risk of cardiovascular disease (6, 7, 12). The consensus of several studies in human subjects is that ethanol exerts its cardioprotective effect by increasing the plasma concentration of antiatherogenic HDL (11, 13–15). The effects of alcohol on other plasma lipoproteins are variable and appear to depend on the amount and duration of alcohol consumption. In normal subjects, moderate intake of alcohol has been reported to have no effect on the transport of very low density lipoprotein (VLDL) triglycerides (16). In alcoholic men with normal liver morphology and function, the lipoprotein pattern has been characterized by normal or subnormal VLDL triglycerides (17). Other studies, however, have reported that high doses of alcohol and chronic alcohol intake are associated with increased serum triglyceride levels (18), elevated phospholipid and cholesterol content of VLDL (19), increased synthesis of VLDL particles (20), and increased plasma VLDL (21). Although alcohol has been shown to stimulate VLDL production in the liver, this response appears to be dose-dependent and occurs at high levels of alcohol intake (20, 22). The concentration of LDL has been reported to increase

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; MEM, minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; ACAT, acyl-coenzyme A:cholesterol acyltransferase; SDS, sodium dodecyl sulfate.

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(21–23), decrease (17), or remain unchanged (16, 19) by alcohol intake.

Several studies have demonstrated that moderate intake of alcohol increases the plasma concentrations of apolipoproteins A-I and A-II (20, 22–25). However, the increase in plasma level of apoA-I was significant only at higher doses of alcohol intake (22) or upon prolonged alcohol consumption (20, 22, 24, 25). The reported effect of alcohol on plasma concentration of apoB in human subjects is variable and ranges from no change (20) to a moderate increase, while the effect on apoE is largely unknown.

The above studies have clearly demonstrated that the effects of ethanol on plasma, as well as nascent lipoproteins and apolipoproteins, are divergent and appear to be both time- and dose-dependent. Further, the mechanisms involved in ethanol-induced changes in the synthesis and secretion of apoA-I- and apoB-containing lipoproteins by the liver are still largely unknown. Initial studies from this laboratory (26) demonstrated that short-term addition of ethanol resulted in a significant increase in the secretion of apoB without affecting that of apoA-I. The purpose of the present study was to determine both the short- and long-term effects of ethanol on the synthesis and secretion of apoA-I- and apoB-containing lipoproteins by human liver using HepG2 cells as an experimental model. In addition, the possible mechanisms involved in ethanol-mediated changes in the synthesis and secretion of apoB-100 including alterations in the cellular content of mRNA, intracellular apoB degradation, modifications in the LDL receptor activity, and potential role of intracellular cholesteryl esters were investigated.

EXPERIMENTAL PROCEDURES

Materials

Minimum essential medium (MEM), trypsin, sodium pyruvate, L-glutamine, and MEM vitamin solution were purchased from Grand Island Biological Company (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Whittaker Bioproducts (Walkersville, MD). Triton X-100, benzamidine, phenylmethylsulfonyl fluoride, leupeptin, bovine serum albumin, diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), and alcohol dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). L-[^{35}S]methionine, [^{32}P]dCTP, and Amplify were obtained from Amersham Corp. (Arlington Heights, IL). Protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. All reagents used for the slab gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Pfizer CP-113,818 was a gift from Pfizer Central Research (Groton, CT).

Cell culture

The human hepatoblastoma cell line HepG2 was obtained from American Type Culture Collection (Rockville, MD). Cells were seeded into tissue culture dishes in MEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, MEM vitamin solution, and 10%

TABLE 1. Evaluation of cytotoxic effects of various concentrations of ethanol

Ethanol Concentration	Total ^{35}S-Labeled Protein		Lactic Dehydrogenase Activity in Mediium
	Medium	Cells	
%	*dpm/mg cell protein × 10^{-4}*		*% of control*
0	90.3 ± 4.1	526.6 ± 44.6	100
0.05	88.7 ± 8.2	547.0 ± 57.9	88 ± 9
0.10	90.3 ± 3.7	570.8 ± 58.7	99 ± 10
0.25	91.2 ± 1.7	552.3 ± 36.4	ND
0.50	89.0 ± 5.4	458.4 ± 20.1	109 ± 36
1.00	75.7 ± 3.1	443.3 ± 21.8	114 ± 6

HepG2 cells were cultured in MEM containing 10% FBS and the indicated concentrations of ethanol. Medium was changed daily and ethanol was added with each medium change. Five days after daily exposure to ethanol, medium was removed, monolayers were washed twice with PBS and serum-free MEM, and the indicated concentrations of ethanol were added. After a 23-h incubation, medium was removed and assayed for lactic dehydrogenase activity. Cells were washed with PBS, scraped off the plates in PBS, sonicated, and assayed for protein content. In experiments where the total newly synthesized protein was measured, the incubation time with serum-free MEM in the presence or absence of ethanol was 5 h. The incorporation of [^{35}S]methionine into cellular and secreted proteins was measured as trichloroacetic acid-precipitable radioactivity. Values are mean ± SD of duplicate dishes. Values for total protein synthesis and secretion are representative of four separate experiments. The protein contents of cells, expressed as mean ± SEM of triplicate dishes, were: 7.5 ± 0.3, 7.5 ± 0.4, 7.2 ± 0.1, 7.1 ± 0.2, 6.9 ± 0.1, and 7.1 ± 0.2 mg/100-mm dish for control, 0.05%, 0.1%, 0.25%, 0.50%, and 1.0% ethanol-treated cells, respectively. ND, not determined.

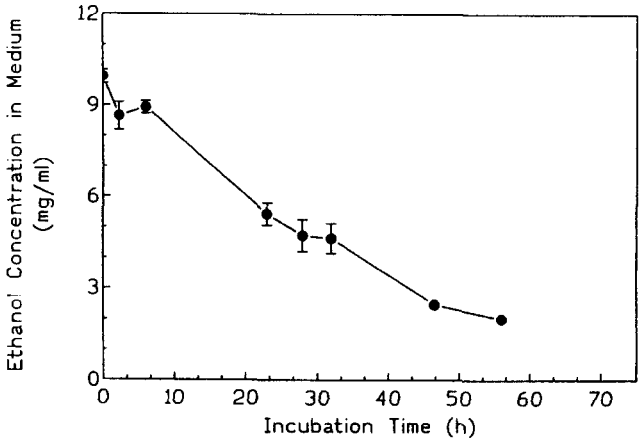

Fig. 1. Rate of ethanol evaporation. Ethanol at a final concentration of 1% was added to MEM in 35-mm dishes without cells. At the indicated time points, medium was removed and assayed for ethanol content as described in the methods. Values are mean ± SEM of triplicate dishes.

fetal bovine serum (FBS) and incubated at 37°C in a 95% air/5% CO_2 atmosphere as previously described (27–29). In all experiments medium was changed 48 h after plating, unless otherwise stated. In experiments where the mass of lipids and apolipoproteins were measured, at the end of incubation, the conditioned medium was removed and, to prevent oxidative and proteolytic damage, a preservative cocktail (30) was added to obtain final concentrations of 500 units/ml penicillin-G, 50 μg/ml streptomycin sulfate, 20 μg/ml chloramphenicol, 1.3 mg/ml ε-amino caproic acid, and 1 mg/ml EDTA. The medium was centrifuged at 2000 rpm for 30 min at 4°C to remove small amounts of cells and debris. The supernatant fraction was concentrated approximately 10- to 15-fold with either polyvinylpyrrolidone (PVP) or sucrose placed outside the dialysis bag (5000 mol wt cut-off). The monolayers were washed three times with PBS, scraped off the plate after addition of 2 × 2 ml of PBS, and sonicated. Aliquots of cell suspension were analyzed for cellular neutral lipids and protein.

Determination of ethanol concentration and lactic dehydrogenase activity in medium

The concentration of ethanol in the medium was determined by the method of Bonnichsen (31). Lactic dehydrogenase activity in the cell culture medium was measured as described by Bergmeyer, Bernt, and Hess (32).

Determination of neutral lipids and apolipoproteins

The concentrations of triglycerides, unesterified cholesterol, and cholesteryl esters in cells and culture medium were determined by gas–liquid chromatography (33). The concentrations of apolipoproteins A-I, A-II, B, and E in the medium were measured by electroimmunoassays using monospecific polyclonal antibodies to these apolipoproteins as described in detail elsewhere (34–36). Polyclonal antisera to apoA-I and apoB were prepared in this laboratory as previously described (34, 35). Polyclonal antisera to apoA-II and apoE were obtained from Dr. Petar Alaupovic. The specificity of antisera was established by double-diffusion analysis, cross immunoelectrophoresis, and electroimmunoassays. These antisera have been used in this laboratory for quantitation of apolipoproteins, immunoprecipitation, and immunoblotting analyses (37, 38). Cell protein was measured by the method of Lowry et al. (39).

De novo synthesis and secretion of apoA-I and apoB-100

To determine the rate of de novo synthesis and secretion of apoA-I and apoB, HepG2 cells were pulse-labeled by incubation with 1 ml of methionine-free medium containing L-[^{35}S]methionine in the presence and absence of ethanol under conditions described for each experiment in the Results. After each incubation, medium was removed, cells were washed three times with cold PBS, and immediately solubilized by adding lysis buffer as described by Dixon, Furukawa, and Ginsberg (40). Protease inhibitor mixture was added to both medium and cell lysate to obtain the final concentration of 5 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units of aprotinin/ml of medium, 1.3 mg/ml ε-aminocaproic acid, 500 units/ml penicillin G, and 5 mg/ml streptomycin sulfate. In addition to the above protease inhibi-

TABLE 2. Short-term effect of ethanol on the net accumulation of lipids in HepG2 cells

Ethanol Concentration	Triglycerides	Unesterified Cholesterol	Cholesteryl Esters
%		*mg/g cell protein*	
0	35.7 ± 1.6	18.4 ± 0.8	12.6 ± 1.0
0.1	36.9 ± 1.7	18.2 ± 0.2	13.0 ± 0.9
0.2	39.3 ± 0.5	18.0 ± 0.1	14.0 ± 0.3
0.5	40.3 ± 1.7	17.7 ± 0.4	13.9 ± 0.8
1.0	46.9 ± 0.2[a]	18.6 ± 0.5	16.7 ± 0.1[b]

Cells were grown in MEM containing 10% FBS. After 4 days in culture, maintenance medium was removed, monolayers were washed twice with PBS and were incubated for 18 h in serum-free MEM in the presence or absence of ethanol. The concentrations of neutral lipids in the cells were determined by gas–liquid chromatography. Values are means ± SEM of triplicate dishes. Total protein contents of cells, expressed as mg/100-mm dish, were: 7.4 ± 0.3, control; 7.6 ± 0.2, 0.1% ethanol; 7.3 ± 0.2, 0.25% ethanol; 7.5 ± 0.1, 0.5% ethanol; 7.2 ± 0.1, 1.0% ethanol.

[a]The difference between control and ethanol-treated cells was significant at $P = 0.002$.

[b]The difference between control and ethanol-treated cells was significant at $P = 0.015$.

TABLE 3. Short-term effect of ethanol on the net accumulation of lipids in HepG2 culture medium

Ethanol Concentration	Triglycerides	Unesterfied Cholesterol	Cholesteryl Esters
%		μg/g cell protein/h	
0	82.7 ± 12.1	80.2 ± 6.6	28.3 ± 2.7
1.0	145.1 ± 12.2[a]	112.8 ± 8.7[b]	40.5 ± 4.2[c]

Cells were grown in MEM containing 10% FBS. After 4 days in culture, maintenance medium was removed, monolayers were washed with PBS and incubated for 16–20 h with serum-free MEM with or without ethanol. The concentrations of neutral lipids in the culture medium were determined by gas–liquid chromatography. Protein contents of cells, expressed as mg/100-mm dish, were: control, 8.5 ± 0.5; ethanol, 8.2 ± 0.4. Values are mean ± SEM of 10 dishes from four separate experiments.

[a]The difference between control and ethanol-treated cells was significant at $P = 0.002$.

[b]The difference between control and ethanol-treated cells was significant at $P = 0.008$.

[c]The difference between control and ethanol-treated cells was significant at $P = 0.025$.

tor mixture, the cell lysate was supplemented with 50 μg/ml leupeptin and 50 μg/ml pepstatin A (40). Aliquots of medium and cell lysate were analyzed for ^{35}S-labeled apoB or apoA-I by immunoprecipitation followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

The [^{35}S]methionine-labeled apoB or apoA-I in cell lysate and secreted into the chase medium were immunoprecipitated using monospecific antibodies to human plasma apoB or apoA-I as described in the following example. Protein A-Sepharose CL-4B was washed according to the manufacturer's instructions. Twelve mg of washed protein A-Sepharose was placed in 1.5 ml microcentrifuge tubes and resuspended in 0.5 ml of buffer A (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% (v/v) NP-40, 0.1% (w/v) SDS, and 0.1% (w/v) bovine serum albumin, and 10 μl of goat antiserum to human plasma apoB or rabbit antiserum to human plasma apoA-I were added. The tubes were rotated for 4 h at room temperature or overnight at 4°C. The bound antibody was removed by centrifugation at 1000 rpm for 2–3 min. The supernatant was discarded and the protein A-Sepharose–antibody complex was washed six times with buffer A. Aliquots (0.5 ml) of medium or cell lysate were added to washed gels and rotated at 4°C overnight. The mixtures were centrifuged as above and the supernatants were discarded. The gels were washed three times with buffer A and three times with buffer B (same as buffer A but without bovine serum albumin), and immunoprecipitates were collected by centrifugation as above. Labeled apoB or apoA-I was eluted in 60 μl of elution buffer as previously described (29). The eluted ^{35}S-labeled apoB was applied to a 2.5–15% polyacrylamide gradient slab gel containing SDS using the Laemmli's buffer system (41). The eluted labeled apoA-I was applied to a 12% polyacrylamide slab gel. After electrophoresis, the gels were stained, destained, dried, and quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Isolation and characterization of cellular RNA, preparation of probes, and DNA-excess solution hybridization assays

RNA was isolated from HepG2 cells by the guanidine thiocyanate method (42). The integrity of all RNA samples was verified by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. Hybridization probes were prepared from human apolipoproteins A-I, A-II, apoB-100, and apoE cDNA fragments subcloned in bacteriophage M13 vectors. Single-stranded cDNA probes for apolipoproteins A-I, A-II, B, and E were prepared as previously described (28, 29, 43, 44). DNA-excess solution hybridization was the method of choice for determination of mRNA abundance based on its high sensitivity and excellent inter- and intra-assay reproducibility. Hybridization assays for determination of the corresponding mRNAs were carried out as previously described (28, 29, 43, 44) using the respective M13–apo cDNA template as hybridization standards to calibrate absolute mRNA concentration (43). All assays were carried out using RNA from triplicate dishes, and four mRNA measurements were made on RNA prepared from each dish, and thus the values represent the

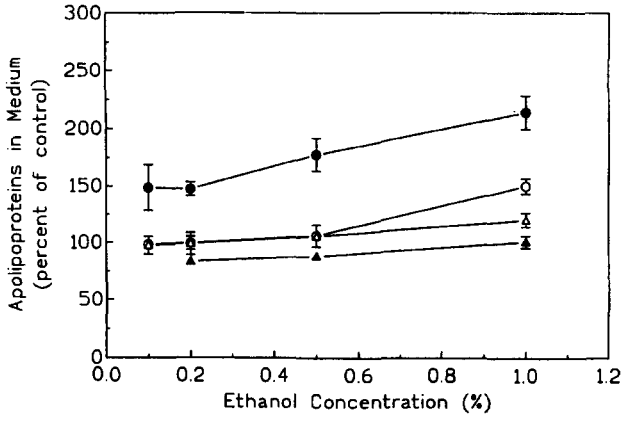

Fig. 2. Ethanol-mediated changes in the net accumulation of apolipoproteins in HepG2 culture medium. Cells were seeded into 100-mm diameter dishes and grown in MEM containing 10% FBS. After 4 days, the maintenance medium was removed, monolayers were washed twice with PBS, and serum-free medium was added. The effects of increasing concentration of ethanol on the net accumulation in the culture medium of apoA-I (△), apoA-II (▲), apoB (●), and apoE (○) after a 16-h incubation were determined. The protein contents of the cells, expressed as mg/dish, were: 8.4 ± 0.3, control; 8.6 ± 0.4, 0.2% ethanol; 7.9 ± 0.4, 0.5% ethanol; 8.0 ± 0.2, 1% ethanol. Values are mean ± SEM of three experiments. The difference between control and 1% ethanol-treated cells was significant at $P = 0.004$ for apoA-I and $P < 0.0001$ for apoE. The differences between apoB concentration in control and 0.1%, 0.2%, 0.5%, and 1.0% ethanol-treated cells were $P = 0.05$, $P = 0.0001$, $P = 0.0006$, and $P = 0.0001$, respectively.

TABLE 4. Short-term effect of ethanol on the net accumulation in HepG2 culture medium and cellular mRNA contents of apolipoproteins A-I, A-II, and E

Ethanol	Accumulation in Medium			Cellular mRNA Content		
	ApoA-I	ApoA-II	ApoE	ApoA-I	ApoA-II	ApoE
%	*μg/g cell protein/h*			*pg/μg RNA*		
0	62.3 ± 4.1 (n=18)	66.6 ± 6.1 (n=9)	68.9 ± 5.6 (n=12)	55.6 ± 1.2 (n=5)	28.8 ± 2.9 (n=5)	28.7 ± 3.7 (n=5)
1.0	73.9 ± 5.2 (n=16)	64.8 ± 7.7 (n=9)	96.1 ± 7.8[a] (n=12)	63.9 ± 3.3[b] (n=5)	28.8 ± 1.3 (n=5)	32.6 ± 3.9 (n=5)

HepG2 cells were grown under experimental conditions described in the legend of Table 3. The concentrations of apolipoproteins were measured by electroimmunoassays. Numbers in parentheses represent total number of dishes. Protein contents of the cells, expressed as mg/100-mm dish, were: control, 8.3 ± 0.9; ethanol-treated, 7.8 ± 0.6. Values for apolipoprotein accumulation in the medium are mean ± SEM of: seven experiments for apoA-I, three experiments for apoA-II, and five experiments for apoE. Values for cellular mRNA content are mean ± SEM of indicated number of dishes from two separate experiments. Four mRNA measurements were made on RNA prepared from each dish.

[a]The difference between control and ethanol-treated cells was significant at $P = 0.0097$.

[b]The difference between control and ethanol-treated cells was significant at $P = 0.0457$.

mean of 12 determinations for both control and treated cells.

Determination of acyl-coenzyme A:cholesterol acyltransferase and LDL receptor activities

The activity of acyl-coenzyme A:cholesterol acyltransferase (ACAT) was determined by incorporation of [1-^{14}C]oleic acid–1.5% bovine serum albumin into cellular and secreted cholesteryl esters as previously described (27, 45). The experiments were performed in the presence and absence of Pfizer CP-113,818 (1 μg/ml of medium), an inhibitor of ACAT (46). LDL (d 1.03–1.063 g/ml) was isolated from plasma of normolipidemic subjects. Plasma LDL were iodinated with Na^{125}I using Iodo-Beads according to the method of Markwell (47), and free iodide was removed by dialysis as previously described (45). The properties of ^{125}I-labeled LDL were similar to those reported previously (45). The binding at 4°C and uptake and degradation at 37°C of ^{125}I-labeled LDL were performed under conditions described in detail elsewhere (45). Control dishes without cells were incubated under identical conditions.

RESULTS

Assessment of cell viability in the presence of various concentrations of ethanol

To examine any potential cytotoxic effect of ethanol, total cellular protein content, total protein synthesis and secretion, lactic dehydrogenase activity in the medium, and the ratio of live to dead cells were determined after 5 days of daily addition of ethanol. As shown in **Table**

TABLE 5. Short-term effect of ethanol on the net accumulation of apoB in culture medium and its cellular mRNA abundance

Exp. No.	Ethanol Concentration	Apolipoprotein B	
		Accumulation in Medium	Cellular mRNA Content
	%	*μg/g cell protein/h*	*pg/μg RNA*
1[a]	0	76.8 ± 6.9	350.5 ± 15.2
1	0.2	120.0 ± 2.1[c]	411.9 ± 16.3[e]
2–4[b]	0	72.2 ± 7.1	310.2 ± 38.9
2–4	1.0	131.2 ± 15.2[d]	389.6 ± 22.2

Cells were grown in MEM containing 10% FBS. After 4 days in culture, maintenance medium was removed, monolayers were washed with PBS and incubated for 16–20 h in serum-free MEM with or without the indicated concentrations of ethanol. Protein contents of cells, expressed as mg/100-mm dish, were: control, 7.5 ± 0.1; ethanol-treated, 7.7 ± 0.2 in experiment 1 and control, 8.8 ± 0.4; ethanol-treated, 8.4 ± 0.3 in experiments 2–4.

[a]Values are mean ± SEM of 4 dishes.

[b]Values are mean ± SEM of 10 dishes from three experiments.

[c]The difference between control and ethanol-treated cells was significant at $P = 0.001$.

[d]The difference between control and ethanol-treated cells was significant at $P = 0.004$.

[e]The difference between control and ethanol-treated cells was significant at $P = 0.003$.

1, ethanol from 0.05% to 1.0% had no significant effect on the total protein content of the cells. Similarly, the total protein synthesis and secretion, measured as trichloroacetic acid-precipitable [^{35}S] in the cells and medium, respectively, were not significantly affected by ethanol (Table 1). The activity of lactic dehydrogenase in the culture medium, a measure of the extent of cell damage by ethanol, was not affected by ethanol concentrations ≤ 0.50%. A small, but not significant, increase in LDH activity in the medium was observed at 1.0% ethanol (Table 1). Similar results were obtained after short-term incubation with ethanol (data not shown). In addition to the above assays, cell viability after a 24-h incubation with 0.05–1.0% ethanol was assessed by fluorescence microscopy using the Live/Dead EukoLight Viability/Cytotoxicity Kit from Molecular Probes, Inc. (Eugene, OR), according to the manufacturer's protocol. Results demonstrated that the cell viability in the presence of 1% ethanol was not significantly different than that observed with 0.05% ethanol (data not shown).

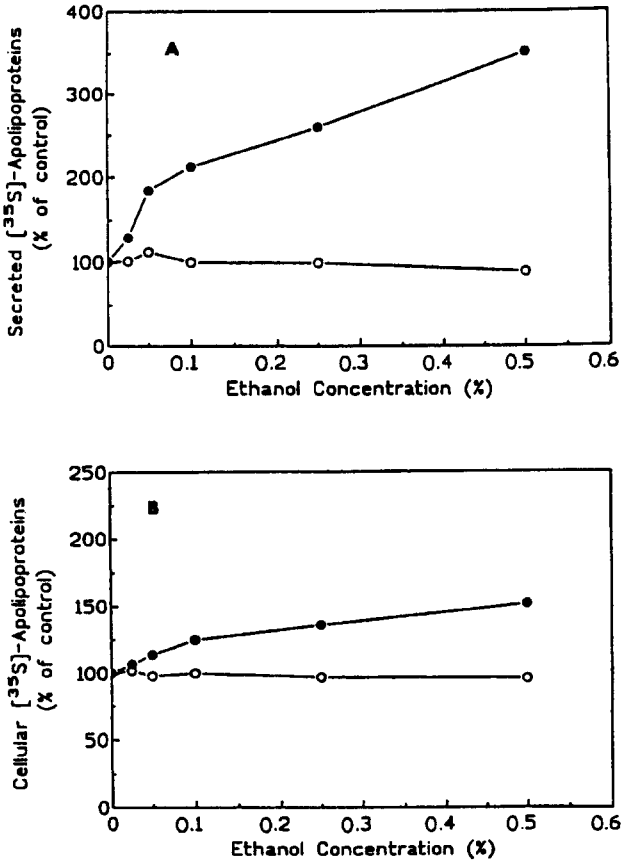

Fig. 3. Short-term effect of ethanol on the synthesis and secretion of apolipoproteins A-I and B. Cells were seeded into 35-mm diameter multiwell dishes in MEM containing 10% FBS. After 5 days in culture, the maintenance medium was removed, cells were washed with PBS, and were incubated with serum-free medium containing [^{35}S]methionine (70 μCi/ml) in the presence or absence of the indicated concentrations of ethanol. After a 6.5-h incubation, medium was removed and ^{35}S-labeled apoA-I (○) and apoB (●) in medium (panel A) and cell lysate (panel B) were immunoprecipitated and subjected to either a 12% (for separation of apoA-I) or a 2.5–15% gradient (for separation of apoB) SDS-polyacrylamide gel electrophoresis as described in Methods. The bands corresponding to apoA-I and apoB-100, based on calibration protein standards and apoB in applied human plasma LDL, were quantified using PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Values are an average of three separate experiments. The protein contents of the cells, expressed as mg/35-mm dish, were: control, 1.8 ± 0.1; ethanol-treated (0.5%), 2.0 ± 0.1. The protein contents of cells incubated with 0.05%, 0.1%, and 0.25% were not different from control values. The differences in secreted apoB between control and 0.025%, 0.05%, 0.10%, 0.25%, and 0.50% ethanol-treated cells were significant at $P = 0.0018$, $P = 0.0012$, $P = 0.0001$, $P = 0.005$, and $P = 0.0003$, respectively. No significant differences were observed for apoA-I synthesis and secretion.

Rate of ethanol evaporation

The rate of ethanol evaporation from the medium in the absence of cells is shown in **Fig. 1.** Within the first 6 h of incubation, the time span used in all studies on the effects of ethanol on the de novo synthesis and secretion of apolipoproteins, only 10% of the initial concentration of ethanol was lost from the system. After a 20-h incubation, the maximum time used in all studies on the net accumulation of lipids and apolipoproteins in the medium, 40% of the initial concentration of ethanol was evaporated (Fig. 1). It required 3.6 days of incubation at 37°C for the complete evaporation of ethanol from the dishes (data not shown). Based on these results, the estimated level of ethanol in the culture medium after a 15–18 h incubation time used in the following studies would be between 74 and 68% of the initial concentration of ethanol.

Short-term effect of ethanol on the accumulation of lipids in HepG2 cells and medium

As shown in **Table 2,** addition of ethanol, at all concentrations tested, had no significant effect on the cellular content of unesterified cholesterol. On the other hand, ethanol caused a gradual increase in the cellular concentrations of triglycerides and cholesteryl esters with the maximum effect occurring at 1% ethanol where a significant increase of 32% was observed in both esterified lipids (Table 2). Analysis of data shown in Table 2 demonstrated that the composition of neutral lipids in cells incubated with 1% ethanol was characterized by a moderately higher triglyceride and cholesteryl ester content.

As the most significant effect of ethanol on cellular content of triglycerides and cholesteryl esters was observed at 1% ethanol, it was reasonable to examine the changes in the secreted lipids under the same experimental conditions. Initial studies (Table 1) demonstrated a lack of any significant difference in cell viability between 0.05% (10.9 mM) and 1.0% (216 mM) ethanol and, thus, justified the use of 1% ethanol in a few studies

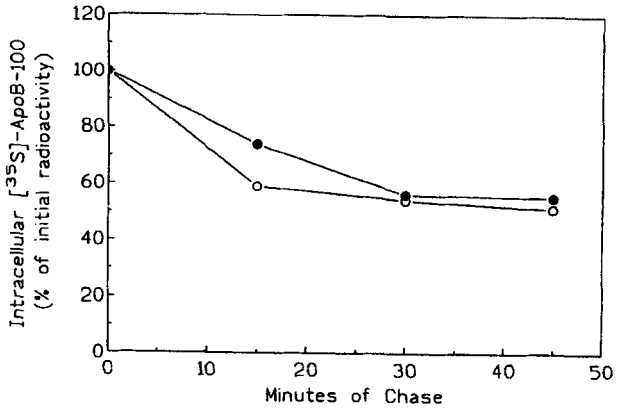

Fig. 4. Short-term effect of ethanol on the intracellular degradation of apoB-100. HepG2 cells were seeded into 35-mm multiwell dishes in MEM containing 10% FBS. After 4 days in culture, the maintenance medium was removed and monolayers were washed twice with serum-free medium and were preincubated for 60 min with the same medium in the presence or absence of 0.5% ethanol. Preincubation medium was removed, cells were pulsed for 30 min with serum-free, methionine-free MEM containing [^{35}S]methionine (80 μCi/ml of medium) in the presence or absence of 0.5% ethanol and chased for 15, 30, 45, and 60 min in the presence or absence of 0.5% ethanol. The ^{35}S-labeled apoB in the cell lysate was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis as described in Methods. The radioactivity in the band corresponding to apoB-100 in the presence (●) or absence (○) of ethanol was quantified using PhosphorImager. Values are mean of two separate experiments.

on the net accumulation of lipids and apolipoproteins in the medium. Short-term administration of 1% ethanol resulted in a marked increase of 75% in the net accumulation of triglycerides in the culture medium (**Table 3**). Under these experimental conditions, the concentrations of unesterified cholestrol and cholesteryl esters in the culture medium of ethanol-treated cells were increased by 40–43% (Table 3). Hence, short-term addition of ethanol resulted in a 56% increase in the net accumulation of total neutral lipids in the medium (Table 3). Analysis of data presented in Table 3 demonstrated that the composition of neutral lipids in the ethanol-treated cell culture medium was characterized by a moderately higher triglyceride content.

Short-term effect of ethanol on the net accumulation of apolipoproteins in the culture medium and cellular abundance of the corresponding mRNAs

The net accumulation of apolipoproteins A-I and E in the culture medium was not significantly affected by short-term incubation of cells with ethanol at concentrations ≤ 0.5% (**Fig. 2**). However, addition of 1% ethanol caused a significant (P = 0.004) increase of 21% in the net accumulation of apoA-I in the culture medium and a more pronounced and significant (P < 0.001) increase of 50% in that of apoE (Fig. 2). By contrast, ethanol caused a dose-dependent increase in the net accumulation of apoB in the culture medium (Fig. 2). A significant (P = 0.05) increase of 48% in apoB production was observed at the lowest concentration (0.1%) of ethanol tested, and a highly significant (P < 0.0001) 2-fold increase occurred at 1% ethanol (Fig. 2). The net accumulation of apoA-II in the medium was not altered by ethanol at all concentrations tested (Fig. 2). In accordance with the unaffected concentration of apoA-II in the medium, there was no change in the mRNA level (**Table 4**). The observed 19% increase in the net accumulation of apoA-I in the medium was paralleled by a 15% elevation (P = 0.046) in its mRNA abundance (Table 4). Short-term addition of 1% ethanol caused a more prominent increase of 40% in the net accumulation of apoE in the culture medium; however, this was accompanied by only a 14% increase in apoE mRNA abundance (Table 4).

The major short-term effect of 1% ethanol was on the net accumulation of apoB in the culture medium that, in a series of 9 experiments, was increased by an average of 91%. Consistent with the enhanced rate of apoB production, the concentration of apoB mRNA was increased in ethanol-treated cells (**Table 5**). However, the 56% and 82% increase in apoB net accumulation in the presence of 0.2% and 1% ethanol, respectively, did not correspond to a 18% and 26% elevation in apoB mRNA abundance under these conditions (Table 5). These results suggest that stimulation of apoB production by short-term addition of ethanol is due, primarily, to alterations in post-transcriptional processes.

Short-term effects of ethanol on the synthesis and secretion of apoA-I and apoB-100

To establish that the ethanol-mediated changes in the net accumulation of apoA-I and apoB in the culture medium (Fig. 2 and Tables 4 and 5) were due to the altered secretion, the incorporation of L-[^{35}S]methionine into newly synthesized and secreted apoA-I and apoB was determined. As shown in **Fig. 3,** short-term addition of ethanol increased the secretion of newly synthesized apoB-100. The effect of ethanol was dose-dependent and was already evident at 0.025% where a significant (P = 0.0018) 29% increase in the secretion of apoB-100 was observed (Fig. 3A). By contrast, the secretion of newly synthesized apoA-I was not significantly affected by short-term addition of ethanol at concentrations ≤ 0.5% (Fig. 3A). Concurrent increase of 14–52% in the intracellular concentration of ^{35}S-labeled apoB as a function of ethanol concentration (Fig. 3B) indicates that ethanol stimulated both the synthesis and secretion of apoB. Similarly, the unchanged level of ^{35}S-labeled apoA-I in the culture medium of ethanol-treated cells (Fig. 3A) was not due to its diminished rate of secretion as the intracellular concentration of ^{35}S-labeled apoA-I was also unchanged (Fig. 3B). Under these experimental

TABLE 6. Role of LDL receptor in the regulation of apoB production by acute ethanol

Preincubation Medium	^{125}I-Labeled LDL (ng/mg cell protein)			
	Binding	Uptake	Degradation	
			Total	Iodide-free
MEM	324.8 ± 8.0	501.4 ± 5.3	3323.6 ± 93.7	224.7 ± 14.4
MEM + ethanol	299.8 ± 13.4	510.3 ± 13.3	3392.1 ± 76.0	214.4 ± 3.9

HepG2 cells were grown in 16-mm diameter multi-well dishes. Four-day-old cells were washed with PBS and were preincubated for 19 h with MEM in the presence or absence of 0.5% ethanol. Preincubation medium was removed, cells were washed twice with PBS, and fresh medium containing ^{125}I-labeled plasma LDL (20 μg protein/ml of medium) was added. The binding at 4°C and uptake and degradation at 37°C of ^{125}I-labeled LDL during a 4.5-h incubation with HepG2 cells were determined. Protein contents of cells, expressed as mg/well, were: control, 0.40 ± 0.01; ethanol-treated, 0.41 ± 0.01. Values are mean ± SEM of triplicate dishes.

conditions, the total protein synthesis and secretion, measured as trichloroacetic acid precipitable radioactivity in the cells and medium, respectively, were not significantly altered by short-term addition of ethanol at 0.025–0.5% (data not shown).

To examine the effect of ethanol on early stages of synthesis and secretion of apoB-100, pulse-chase experiments were performed. Experiments were carried out as described in the legend to **Fig. 4** which demonstrates the average results of two experiments. A small amount of apoB-100 was first detected in the culture medium of both control and ethanol-treated cells after 30 min of chase but it could be accurately measured after 45 min of chase (data not shown). At the end of pulse period

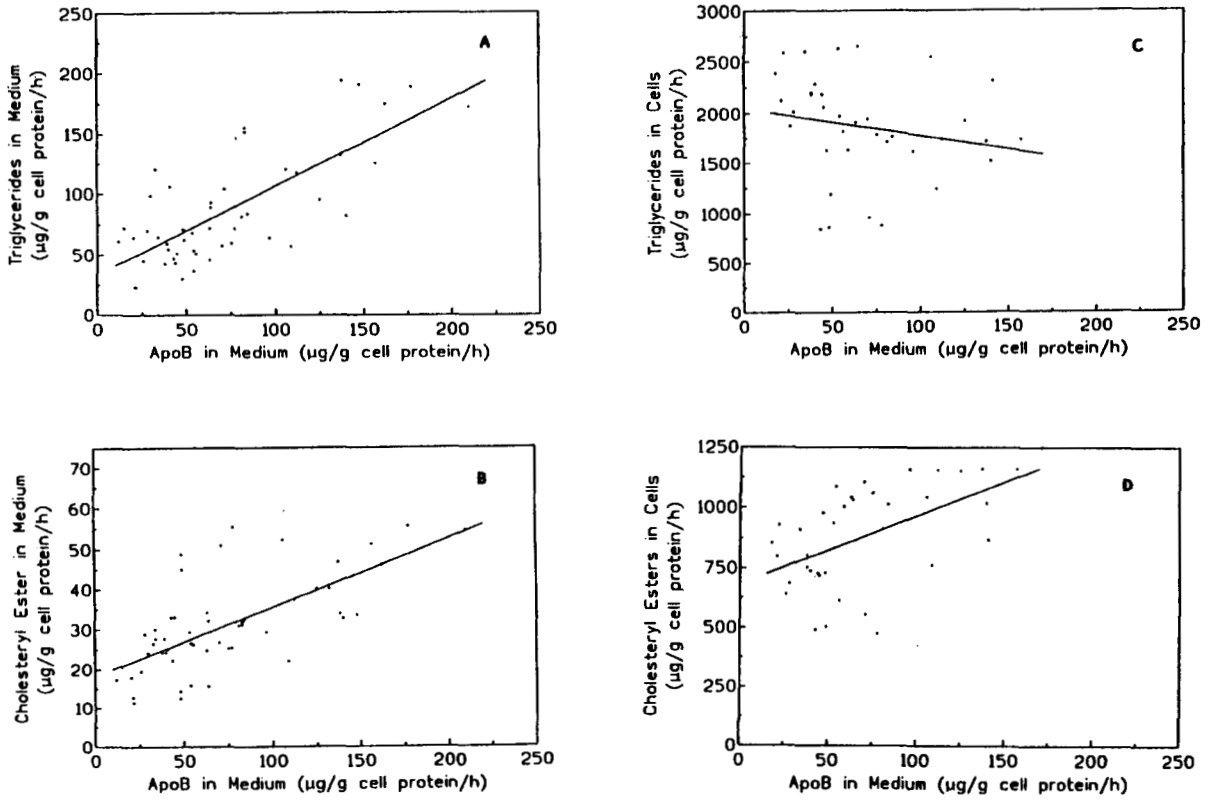

Fig. 5. Relationship between the rate of apoB production and the net accumulation of triglycerides and cholesteryl esters in HepG2 cells and culture medium. The data from eight experiments with 52 samples were used to establish the relationship between apoB and triglycerides and cholesteryl esters in the culture medium. Panel A shows the correlation (r = +0.74, P < 0.0001) between the net accumulation of apoB and triglycerides in the medium. Panel B shows the correlation (r = +0.67, P < 0.0001) between the net accumulation of apoB and cholesteryl esters in the medium. The data from four experiments with 36 samples were used to establish the relationship between apoB in the medium and cellular triglycerides and cholesteryl esters. Panel C shows the correlation (r = -0.21, P = 0.23) between the net accumulation of apoB in the medium and cellular content of triglycerides. Panel D shows the correlation (r = 0.51, P = 0.0014) between the net accumulation of apoB in the medium and cellular content of cholesteryl esters.

TABLE 7. Effect of acyl-CoA:cholesterol acyltransferase inhibitor, Pfizer CP-113,818, on the synthesis and secretion of lipids and apolipoprotein B in HepG2 cells

Additions	Cellular Lipids			Secreted Lipids			
	PL	TG	CE	PL	TG	CE	ApoB-100
	nmol/mg cell protein			mol/mg cell protein			*% of control*
Ethanol	59.20 ± 1.01	223.51 ± 10.63	8.59 ± 0.04	0.97 ± 0.01	4.13 ± 0.60	0.26 ± 0.01	100
CP-113,818 (1 μg/ml)	58.39 ± 0.35	219.35 ± 5.95	0.79[a] ±0.18	1.52[b] ± 0.18	4.18 ± 0.31	0.08[c] ± 0.03	76[d] ± 7

HepG2 cells were grown in 35-mm diameter multiwell dishes in MEM containing 10% FBS. After 4 days in culture, the maintenance medium was removed, cells were washed with PBS and 1.5 ml of serum-free MEM containing [1-14C]oleic acid (0.4 mM bound to 1.5% bovine serum albumin) was added to each well. Pfizer CP-113,818 was dissolved in ethanol and added at the final concentration of 1 μg/ml of medium; the concentration of ethanol in all dishes was 0.5%. The incorporation of [1-14C]oleic acid into phospholipids (PL), triglycerides (TG), and cholesteryl esters (CE) after a 16-h incubation was determined as described in Methods. To determine the effect of Pfizer CP-113,818 on the synthesis and secretion of apoB-100, maintenance medium was removed, cells were washed with PBS, and incubated for 16–20 h in serum-free MEM in the presence or absence of Pfizer CP-113,818. The preincubation medium was removed, methionine-free MEM containing [^{35}S]methionine (50 μCi/ml) was added, and cells were incubated for 3 h in the presence or absence of Pfizer CP-113,818. The concentration of ^{35}S-labeled apoB-100 was measured as described in Methods. Values for lipids are mean ± SD of duplicate dishes; values for apoB are mean ± SEM of 3 dishes. The protein contents of cells, expressed as mg/35-mm dish, in the absence and presence of CP-113,818 were 1.53 ± 0.02 and 1.59 ± 0.06, respectively.

[a]The difference between ethanol- and CP-113,818-treated cells was significant at $P = 0.0003$.

[b]The difference between ethanol- and CP-113,818-treated cells was significant at $P = 0.027$.

[c]The difference between ethanol- and CP-113,818-treated cells was significant at $P = 0.015$.

[d]The difference between ethanol- and CP-113,818-treated cells was significant at $P = 0.026$.

(0-time chase time point), there was no significant difference in the amount of ^{35}S-labeled apoB-100 immunoprecipitated from control (93,386 pixels) and ethanol-treated cells (91,119 pixels). After 15 min of chase, before any apoB-100 could be detected in the medium, the concentration of ^{35}S-labeled apoB-100 in cell lysate in the control and ethanol-treated cells was 59% and 74%, respectively, of the initial value (Fig. 4). However, this difference, which was not statistically significant, became negligible at 30 and 60 min of chase (Fig. 4). These results suggest that the higher number of counts in ethanol-treated cells after the 15-min chase might be due, in part, to a modest decrease in the early intracellular degradation of apoB-100. However, as no apoB was secreted during the first 15-min of chase, this difference may reflect the higher amount of ^{35}S-labeled apoB-100 in the secretory pathway of ethanol-treated cells that will lead to secretion.

Role of LDL receptor in the regulation of apoB production by ethanol

The above studies demonstrated that the increase in the accumulation of apoB-100 in the culture medium of HepG2 cells after short-term incubation with ethanol was primarily due to increased secretion of the newly synthesized apoB. However, down-regulation of LDL receptor and subsequent decrease in the catabolism of secreted lipoproteins under the present experimental conditions may also play a role in this process. As shown in **Table 6,** a 19-h preincubation of HepG2 cells with 0.5% ethanol had no significant effect on the binding at 4°C, and uptake and degradation at 37°C of ^{125}I-labeled plasma LDL. These results indicated that under these experimental conditions, ethanol did not alter the activity of the LDL receptor in HepG2 cells. Similar results were obtained when cells were preincubated with 0.25% ethanol (data not shown). Subsequent experiments demonstrated that the re-uptake of nascent apoB-containing lipoproteins by HepG2 cells was minimal and that this was not altered by short-term incubation with 0.5% ethanol (data not shown).

Correlation between the concentrations of cellular and secreted triglycerides and cholesteryl esters and the net accumulation of apoB in the medium

Correlation analyses were performed to determine possible relationship between the ethanol-mediated changes in the concentrations of triglycerides and cholesteryl esters in the medium and the rate of apoB secretion. Results from experiments shown in Tables 2, 3, and 5 and Fig. 2, showed that after a 16–20 h short-term incubation with 0.1–1% ethanol, there were significant positive correlations between the net accumulation in the medium of apoB and triglycerides ($r = +0.74$, $P < 0.0001$) (**Fig. 5A**), and apoB and cholesteryl esters ($r = +0.67$, $P < 0.0001$) (Fig. 5B). Previous studies from this laboratory (29) demonstrated that the rate of apoB production was not correlated with the triglyceride mass in the cells but was positively correlated with the cellular cholesteryl esters. Similar results were obtained in the present study. There was no correlation ($r = -0.205$, n =

36) between the cellular content of triglycerides and the rate of apoB accumulation in the medium (Fig. 5C). By contrast, a significant positive correlation (r = +0.5113, P = 0.0014, n = 36) was observed between the concentration of cholesteryl esters in the cells and the net accumulation of apoB in the medium (Fig. 5D). To further establish the potential role of cellular cholesteryl esters in the regulation of apoB synthesis and secretion, the effect of Pfizer CP-113,818, an inhibitor of ACAT (46), on these processes was studied. Addition of CP-113,818 (1 μg/ml of medium) had no effect on the total protein synthesis and secretion, measured as trichloroacetic acid-precipitable [^{35}S] in the cells and medium, respectively, indicating lack of cytotoxic effect of this compound. Incubation of cells with CP-113,818 caused a significant reduction in the incorporation of [1-^{14}C]oleic acid (specific activity of 1500 dpm/nmol) into cellular (91%) and secreted (69%) cholesteryl esters, without affecting those of triglycerides (**Table 7**). The secretion of apoB, measured by the incorporation of L-[^{35}S]methionine into immunoprecipitated apoB-100, was also decreased by a significant (P = 0.026) 24% (Table 7). The inhibitory effect of CP-113,818 was observed in the presence and absence of oleate. The disproportionate decrease in the secretion of apoB and cholesteryl ester might be due to the experimental procedure used as ACAT activity rather than cholesteryl ester mass was measured. The effect of CP-113,818 on the association between apoB secretion and the mass of cholesteryl ester in the cells and that secreted into the medium remains to be established.

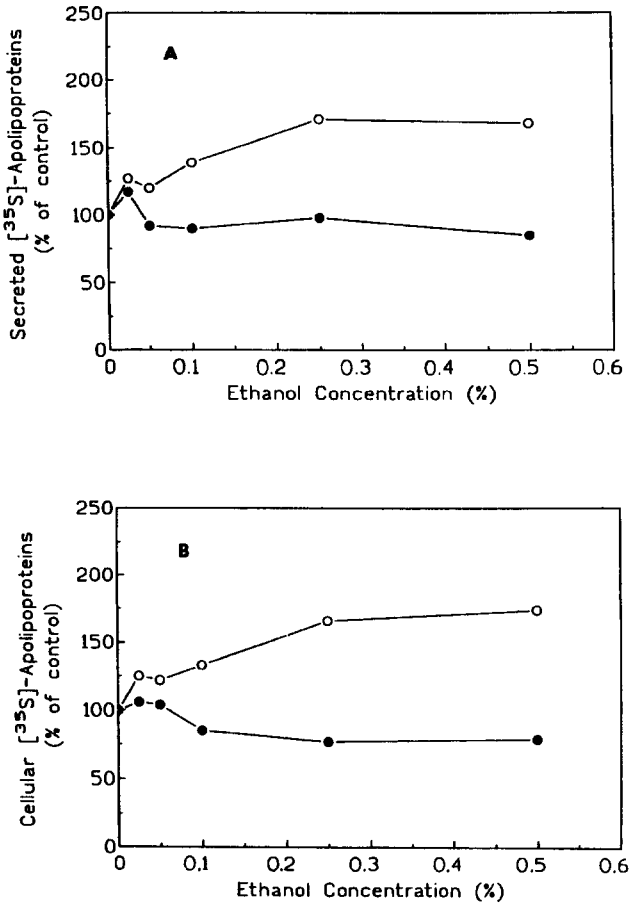

Fig. 6. Long-term effect of ethanol on the synthesis and secretion of apolipoproteins A-I and B. Cells were seeded into 35-mm diameter multiwell dishes in MEM containing 10% FBS and the indicated concentrations of ethanol. Medium was changed daily and ethanol was added with each medium change. After 5 days in culture, medium was removed and cells were incubated with serum-free medium containing [^{35}S]methionine (70 μCi/ml) in the presence or absence of ethanol. Long-term effect of increasing concentration of ethanol on the incorporation of [^{35}S]methionine into secreted (panel A) and cellular (panel B) apoA-I (○) and apoB (●) after a 6.5-h incubation was determined. Values are an average of three separate experiments. The protein contents of the cells, expressed as mg/35-mm dish, were: 2.22, 2.16, 2.12, 2.10, 2.22, and 2.30 for control, 0.025%, 0.05%, 0.1%, 0.25%, and 0.5% ethanol-treated cells, respectively. The differences in apoA-I secretion between the control and 0.10%, 0.25%, and 0.50% ethanol-treated cells were significant at P = 0.005, P = 0.0002, and P = 0.018, respectively. The difference in cellular apoA-I levels between the control and 0.10% ethanol-treated cells was significant at P = 0.05. No significant differences were observed in apoB synthesis and secretion between control and ethanol-treated cells.

Long-term effects of ethanol on the synthesis and secretion of apoA-I and apoB-100

The above studies demonstrated that short-term addition of ethanol to HepG2 cells caused a marked increase in the synthesis and secretion of apoB-100 without significantly altering those of apoA-I (Fig. 3). To assess the long-term effects of ethanol on the above processes, HepG2 cells were seeded and grown for 5 days in medium containing 0.025–1.0% ethanol. Medium from both control and ethanol-treated cells was changed daily, and ethanol was added with each medium change. This daily replacement of medium was necessary to maintain the desired concentration of ethanol in the system. Earlier studies showed that approximately 40% of the added ethanol was evaporated during a 20-h incubation (Fig. 1). Therefore, a daily addition of ethanol to cells for 5 days, without changing the culture medium, would result in concentrations of ethanol well above those intended for this series of experiments. Long-term addition of ethanol did not have any significant effect on the total protein synthesis and secretion, measured as trichloroacetic acid-precipitable radioactivity in the cells and medium, respectively. As shown in **Fig. 6,** the rate of incorporation of [^{35}S]methionine into apoB-100 was not affected in response to long-term addition of 0.025%–0.25%, but decreased by a modest 15% after addition of 0.5% ethanol (Fig. 6A). Parallel alterations were observed in the intracellular ^{35}S-labeled apoB-100 (Fig. 6B). By contrast, long-term addition of ethanol stimulated the secretion of apoA-I which was already evident at 0.025% of ethanol (Fig. 6A). The

increase in apoA-I secretion was dose-dependent and ranged from 27% at 0.025% ethanol to 68% in the presence of 0.5% of ethanol (Fig. 6A). The increase in apoA-I secretion was significant at ethanol concentrations ≥ 0.10%. The ethanol-mediated changes in the cellular ^{35}S-labeled apoA-I (Fig. 6B) were similar to those observed in the medium (Fig. 6A). These results demonstrated that long-term addition of ethanol markedly increased both the synthesis and secretion of apoA-I without significantly affecting those of apoB-100.

DISCUSSION

Ethanol metabolism in HepG2 cells appears to occur primarily through non-alcohol dehydrogenase (ADH) pathway because these cells have low ADH activity (48). The microsomal ethanol oxidizing system (MEOS), which is distinct from ADH and is dependent on cytochrome P-450 (49), has been shown to be active in HepG2 cells (50). MEOS has a relatively high K_m for ethanol compared with ADH, and unlike ADH, the activity of MEOS increases during chronic alcohol consumption (51). Therefore, whereas the major bulk of ethanol at a low blood ethanol concentration is oxidized through the ADH pathway, the MEOS may play a highly significant role in the in vivo ethanol oxidation at high levels and/or chronic intake of alcohol (51) and could explain a number of complications that develop in alcoholics (51). Thus, the relevance of the HepG2 cell model to the in vivo effects of ethanol relates particularly to the important portion of ethanol metabolism in normal liver through MEOS (49, 51).

In the present study, short-term addition of ethanol resulted in a gradual increase in the cellular content of triglycerides and cholesteryl esters which reached the maximum at 1% ethanol without affecting that of unesterified cholesterol. These results are similar to studies in cultured rat hepatocytes (52) and rat hepatoma cell line (53). Although the concentration of ethanol required to elicit the above changes in cellular and secreted neutral lipids was high, ethanol concentration in the portal circulation might be much higher than that in the peripheral blood after alcohol ingestion because of the apparent stimulatory effect of ethanol on hepatic blood flow (21). As in the cells, the concentrations of both triglycerides and cholesteryl esters in the medium were increased by short-term addition of 1% ethanol. In rat hepatocytes, ethanol has been shown to promote fatty acid synthesis (21), to decrease the rate of fatty acid oxidation (54), and to enhance fatty acid esterification (21, 52, 53). These results suggest that ethanol enhances the levels of acetyl-CoA which is a precursor for fatty acid and cholesterol synthesis. This will increase the concentrations of triglycerides and cholesteryl esters. The increase in the net accumulation of unesterified cholesterol in the medium, under conditions where its cellular content was unchanged, might be due to an increase in its direct secretion and/or its enhanced conversion to cholesteryl esters and subsequent secretion as a component of lipoprotein particles. Both pathways would be compensatory mechanisms to maintain cellular cholesterol homeostasis in the event of potential ethanol-mediated excess of cellular unesterified cholesterol. The possible involvement of the above mechanisms in ethanol-mediated changes in cellular and secreted triglycerides and unesterified and esterified cholesterol in HepG2 cells remains to be established.

The ethanol-mediated increase in cellular and secreted triglycerides and cholesteryl esters in response to the short-term addition of ethanol was associated with a marked enhancement in the net accumulation of apoB in the medium. The term production, used herein, refers to the combined processes of synthesis and secretion and is measured by the rate of accumulation in the medium. The rate of apoB production was correlated positively with secreted triglycerides and cholesteryl esters and the cellular content of cholesteryl esters but not with cellular triglycerides. Support for the potential regulatory role of cellular cholesteryl esters in apoB production was provided by studies demonstrating a partial inhibition in apoB production by Pfizer CP-113,818, a potent inhibitor of ACAT (46). Results shown in Fig. 2 demonstrate an increase of 80% in apoB production during a 16-h incubation of HepG2 cells with 0.5% ethanol. Data presented in Table 7 show that, under similar experimental conditions, CP-113,818 diminished the stimulatory effect of ethanol on apoB production by 24%. From these combined results, the calculated contribution of cholesteryl esters to the ethanol-mediated 80% increase in apoB production would be 43%. The present results are consistent with studies by Cianflone et al. (55) in HepG2 cells and those by Marzetta et al. (46) in rabbits and suggest that cellular cholesteryl esters play a partial regulatory role in the secretion of apoB-containing lipoproteins. However, recent studies by Wu et al. (56) have suggested that apoB secretion is not regulated by changes in either the synthesis or the mass of cholesteryl esters. The identification of exact factors responsible for the differences between the present study and that reported by Wu et al. (56) requires further investigation. The above data suggest that a single high dose of ethanol augmented the secretion of triglyceride-rich, apoB-containing lipoproteins with their complement of free and esterified cholesterol. As density classes were not isolated, it is not clear what portion of neutral lipids was associated with apoB. However, our previous studies (27) have shown that 86% of triglycerides and 64% of unesterified and

esterified cholesterol are secreted in d < 1.063 g/ml. These results are consistent with in vivo studies in human subjects demonstrating that the ingestion of high amounts of alcohol increases the synthetic rate of VLDL triglycerides (20).

The increase in the net accumulation of apoB in the medium was due primarily to augmented secretion of newly synthesized apoB-100 which was evident at low concentration (0.05% or 10 mM) of ethanol. The concomitant increase in the cellular concentration of ^{35}S-labeled apoB-100 indicated that ethanol increased both the synthesis and secretion of apoB-100. As apoB mass in the medium and its mRNA level were measured precisely, the disproportionate increase in apoB production and its mRNA abundance suggests that in HepG2 cells, the regulation of apoB synthesis and secretion by short-term addition of ethanol occurs predominately at post-transcriptional levels. The pulse-chase studies showed that short-term addition of ethanol led to a higher incorporation of [^{35}S]methionine into cellular apoB-100 early in the chase. This may result from either an early, but transient, decrease in intracellular degradation of apoB, or from increased synthesis of peptides that were not completed at the end of the pulse period. The rate of intracellular degradation of apoB has been shown to play a significant regulatory role in hepatic apoB secretion (40, 57). Although the role of intracellular degradation cannot be ruled out, our results may also be explained by increased synthesis of apoB. Changes in the translational efficiency of mRNA have been shown to contribute to the insulin-mediated decrease in apoB secretion (58).

The effect of ethanol on apoA-I production was different from that on apoB. The synthesis and secretion of apoB were markedly stimulated by short-term incubation of HepG2 cells with low concentrations of ethanol while those of apoA-I were minimally increased only at high level of ethanol. By contrast, long-term addition of low concentrations of ethanol produced a large increase in the synthesis and secretion of apoA-I and no major change in those of apoB. These results on the production of apoA-I are similar to studies by other investigators (59, 60) demonstrating enhanced secretion of apoA-I after prolonged incubation of cells with low concentration of ethanol. Our results on the long-term effect of ethanol on apoB production are similar to that reported by Wang, Byrne, and Hales (61). Similar results have been observed in human subjects (17, 20, 22, 24, 25, 62, 63). The results of the present study on the short- and long-term effects of ethanol on the synthesis and secretion of apoA-I and apoB strongly support the concept that the effect of ethanol is both time- and dose-dependent and is different for these two apolipoproteins.

The molecular mechanisms underlying the observed divergent effects of ethanol on the synthesis and secretion of apoA-I and apoB are unknown. Ethanol has been shown to cause major changes in microsomal function, e.g., the activities of drug-hydroxylating enzymes and the overall clearance of drugs in human subjects and animals (64). Whereas acute administration of ethanol decreases the activities of these enzymes by competitive inhibition and binding to cytochrome P-450, chronic consumption of ethanol causes proliferation of endoplasmic reticulum and accompanying increases in cytochrome P-450, which has a high affinity for ethanol in HepG2 cells (50). In humans, liver cytochrome P-450 concentrations were found to be directly proportional to plasma HDL-cholesterol (65). Additionally, chronic alcohol consumption increases phospholipids and apoA-I in the liver (21, 50), and serum HDL-cholesterol, phospholipids, and apoA-I concentrations in humans (65). To put the results of the present study into perspective, it can be speculated that short-term exposure of cells to ethanol would not affect apoA-I but would enhance the synthesis of triglycerides, unesterified cholesterol, and cholesteryl esters by potential mechanisms described earlier. The concomitant increase in apoB synthesis would be necessary for transport of these lipids out of the cells in the form of nascent VLDL or LDL (27, 37) and thus, preventing lipid accumulation in hepatocytes. On the other hand, long-term administration of ethanol would induce proliferation of endoplasmic reticulum, increase cytochrome P-450 level, and thus, stimulate the synthesis of phospholipids and cholesterol. An increase in apoA-I would be necessary to secrete these lipids in the form of nascent HDL which contain a high content of cholesterol and phospholipids (38, 66). It is possible that under these conditions, apoB synthesis could be decreased due to changes in endoplasmic reticulum, a site of its regulation (57) or by damage caused by high levels of acetaldehyde generated by oxidation of ethanol.

The relevance of the present results obtained with HepG2 cells to the pathological effects of ethanol in vivo relates to numerous studies (67) indicating that light to moderate alcohol consumption is associated with lower mortality from CHD, and that heavy drinking may actually increase the risk of CHD. Approximately 45% of the cardioprotective effect of ethanol is due to increased HDL concentration (68). Studies by Gruchow et al. (6) have demonstrated that regular drinkers with consistent daily moderate intake of alcohol have lower triglycerides and LDL-cholesterol, higher HDL-cholesterol, and less coronary artery occlusion compared with regular drinkers with sporadic alcohol intake. The picture that emerges from numerous studies (67) is that while regu-

lar light to moderate alcohol consumption increases HDL and protects against CHD, heavy drinking, both acutely and chronically, is associated with an increased risk of CHD. The changes in lipids, apoA-I, and apoB observed in the present study are consistent with the above findings relating to the ethanol-mediated effects on lipoproteins and risk of CHD in humans. The consistency of the results observed in this study with those of lipoproteins and CHD risk in humans suggests that the HepG2 cell line may be a useful model to further examine the molecular mechanisms of ethanol effects on hepatic lipoprotein synthesis and secretion.

We would like to thank Dr. Petar Alaupovic for providing polyclonal antibodies to apolipoproteins A-II and E. The cDNAs for apoA-I, apoA-II, apoB, and apoE were kindly provided by Dr. David L. Williams with permission of Dr. J. Breslow, Dr. A. Protter, and Dr. H. Bryan Brewer. The technical assistance of Particia Otto and Ben Guo is gratefully appreciated. The assistance of Rachel Spain in preparing this manuscript is acknowledged. This investigation was supported by research grant HL 46178 from the National Institutes of Health and by the resources of The University of Alabama at Birmingham.

Manuscript received 11 October 1995 and in revised form 11 January 1996.

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