Effects of chronic alcohol treatment on the synthesis, sialylation, and disposition of nascent apolipoprotein E by peritoneal macrophages of rats[1–3]

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ABSTRACT

Background: Plasma apolipoprotein (apo) E, a sialoprotein, plays an important role in reverse cholesterol transport. Previously, we showed that chronic alcohol consumption impairs glycosylation of apo E in rat liver. Peritoneal macrophages are another significant apo E synthesis site.

Objective: The main purpose of this study was to determine the effects of chronic alcohol feeding of rats on the synthesis, sialylation, and sialic acid content of macrophage apo E and its ability to bind to the HDL_3 molecule in vitro.

Design: Rats were fed an alcoholic diet or an isoenergetic control diet for 8 wk, after which peritoneal macrophages isolated from them were cultured and analyzed for apo E metabolism.

Results: Macrophages from alcohol-fed rats accumulated 33.3% more ($P < 0.05$) cholesterol than did those from control rats when incubated with acetylated LDL. These macrophages showed a 51–57% lower relative sialylation rate of apo E ($P < 0.001$) but no significant difference in relative protein synthetic rate. The sialic acid content of the intracellular and secreted forms of apo E was reduced by 41.8% ($P < 0.001$) and 50.3% ($P < 0.001$), respectively, with chronic alcohol treatment. Secretion of newly synthesized apo E was impaired by 53.7% ($P < 0.001$) and 26.1% ($P < 0.001$) in the absence and presence of HDL in the medium, respectively. Macrophages of alcohol-treated rats secreted apo E with 47.6–67.2% lower ($P < 0.001$) HDL_3 binding ability; binding ability was restored completely by resialylation of the desialylated apo E.

Conclusion: In rats, an alcohol-mediated decrease in sialylation rate resulting in loss of sialic acid residues in apo E impairs the ability of apo E to bind to HDL and consequently in defective reverse cholesterol transport. *Am J Clin Nutr* 2000;72:190–8.

KEY WORDS Chronic ethanol feeding, macrophages, apolipoprotein E, glycosylation, sialylation, sialic acid content, high-density lipoproteins, HDL, reverse cholesterol transport, binding, atherosclerosis, rats, alcohol, coronary heart disease

INTRODUCTION

The influence of alcohol on development of coronary heart disease (CHD) is complex and may be beneficial or harmful depending on the amount of alcohol consumed. The benefits and risks of moderate alcohol consumption in relation to the development of CHD have been reviewed (1). Thus, compared with abstinence, moderate alcohol intake may be beneficial, whereas heavy intake is harmful. The risks of coronary events as a result of heavy drinking have been shown (2).

Epidemiologic evidence has established the protective role of HDLs against atherosclerosis. Plasma HDL-cholesterol concentration is inversely correlated with the incidence of CHD (3). Changes in plasma lipids and lipoproteins associated with alcohol-induced toxicity that result in altered synthesis and metabolism of lipoproteins both in liver and peripheral tissues were reviewed earlier (4, 5).

Plasma apolipoprotein (apo) E is a component of several plasma lipoproteins, including chylomicrons, VLDLs, intermediate-density lipoproteins, and subfractions of LDL and HDL. Basu et al (6) established the role of apo E in the reverse cholesterol transport process. Furthermore, they showed that cholesterol-loaded mouse macrophages and human monocytes are capable of synthesizing and secreting apo E. Plasma apo E is an O-type sialoglycoprotein that has different isoforms depending on the presence of different amounts of sialic acid on its carbohydrate chain (7). Sialic acid residues in apo E, synthesized and secreted by macrophages, may be responsible for the structural conformity and stability of apo E, a prerequisite for the attachment of apo E to phospholipids to form lamellar discs for secretion, as well as recognition of apo E by HDL_3.

We showed earlier that chronic alcohol intake impairs both glycosylation and secretion of apo E from the liver in rats (8).

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Furthermore, we identified specific glycosylation sites in rat liver that are susceptible to attack by alcohol (5). We also showed that although the total plasma apo E concentration did not change with chronic alcohol intake, the proportion of apo E associated with HDL decreased with a concomitant increase in VLDL-associated apo E (9). Because macrophages are another source of plasma apo E, it was pertinent to determine whether the synthesis and glycosylation of apo E was also affected in this tissue and to test whether the association of apo E with plasma HDL_3 was altered as a result of chronic alcohol exposure. Therefore, we pursued studies that explored whether chronic alcohol exposure has any influence on *1*) the synthesis, sialylation, and secretion of apo E by macrophages; *2*) any alterations in the sialic acid content of intracellular and secreted apo E synthesized by macrophages; and *3*) any impairment in the functional ability of secreted apo E to integrate within the HDL_3 molecule.

MATERIALS AND METHODS

Materials

L-[U-^{14}C]Leucine (specific activity: 11.1 MBq/mmol, or 300 mCi/mmol) and [6-^{3}H]*N*-acetyl-D-mannosamine (ManNAc; specific activity: 262.7 GBq/mmol, or 7.1 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc (St Louis). Antiserum to rat apo E was developed in New Zealand white rabbits in our laboratory and was proven to be monospecific, as evidenced by Ouchterlony analysis (10). CNBr-activated Sepharose 4B used as a matrix for the preparation of an anti-apo E affinity column was purchased from Pharmacia (Uppsala, Sweden). Other chemicals, including authentic standards, were from Sigma Chemical Co (St Louis). Dulbecco Minimum Essential Medium (DMEM, henceforth referred to as the medium) for cell culture was purchased from Gibco BRL (Rockville, MD). Hydrofluor scintillation fluid was purchased from National Diagnostics (Manville, NJ). Organic solvents were purchased from Fisher Scientific (Columbia, MD) and were of analytic grade. All separation and purification columns were purchased from Amersham Pharmacia Biotech, Inc (Piscataway, NJ).

Experimental animals

The use of animals involved in this study was approved by the Institutional Animal Care and Use Committee of the Veterans Administration Medical Center, Washington, DC. Weanling, male Wistar rats (Charles River, Wilmington, MA) were maintained with a standard, unpurified diet until they reached a body weight of ≈150 g. They were then divided into 2 groups and pair fed the control or alcohol diet for 8 wk. The diets were isoenergetic and were formulated according to the modified method reported by Lieber and DeCarli (11). Accordingly, 40% of the total energy of the alcohol diet was from fat, 20% from protein, 36% from alcohol, and 4% from carbohydrate. The control diet had an equal amount of dextrimaltose in place of alcohol.

Cell culture

Rat peritoneal macrophages were collected from animals of the control and alcohol groups after an intraperitoneal injection of 5.0 mL sterile saline solution. All subsequent procedures were carried out under sterile conditions, as described previously (12). Briefly, cells were plated and incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 until they reached 90% confluency (≈6×10^6 adherent cells/dish in ≈72 h; nonadherent cells were washed away). All subsequent experiments were carried out with these cultures of confluent cells.

Cholesterol loading of macrophages

The cells in culture were loaded with cholesterol by an overnight incubation with acetylated rat LDL + VLDL fraction (henceforth referred to as the LDL fraction) at 100 mg/L in medium containing 10% fetal bovine serum (FBS). All experimental incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Experiment 1: effects of chronic alcohol intake on apo E synthesis and glycosylation

The cholesterol-loaded rat peritoneal macrophages were incubated in the absence (nonstimulated group) and presence (stimulated group) of rat HDL_3 in leucine-free medium without FBS. L-[U-^{14}C]Leucine (74 GBq, or 2 μCi/dish), [6-^{3}H]ManNAc (37 GBq, or 1 μCi/dish), and unlabeled L-leucine (25 μmol/L) were added to a total of 5 mL medium added to nonstimulated or stimulated rat peritoneal macrophages (6×10^6 adherent cells) from the control and alcohol groups. The culture dishes were placed in a shaker (on low) inside an incubator for 60 min. The reaction contents were then immediately transferred into tubes and centrifuged at 500 × *g* for 5 min, and the medium was discarded. The cells were resuspended and washed 3 times with 10 mmol phosphate-buffered saline (PBS)/L and processed for subcellular fractionation. A 3% (wt:vol) homogenate of macrophage cells was made in sodium phosphate buffer (200 mmol/L, pH 7.0, containing 10 mmol EDTA/L, 1% trasylol, and 1% leupeptin) with 500 mmol sucrose/L. A known amount of homogenate was processed for the isolation of microsomes and a Golgi apparatus–rich fraction (henceforth referred to as the Golgi fraction) as described previously (6, 9). The whole cell homogenate, microsomes, and the Golgi fractions were then analyzed for incorporation of labeled leucine and labeled ManNAc into immunoprecipitable apo E according to our published method (6). The total protein content of the fractions was determined by using the method of Lowry et al (13).

Experiment 2: effects of chronic alcohol intake on kinetics of apo E secretion

Four sets each of peritoneal macrophage cultures (6×10^6 adherent cells) from rats of the control and alcohol groups, overloaded with cholesterol and maintained on nonstimulated and stimulated media in a total volume of 5 mL as described above, were incubated for 1 h with L-[U-^{14}C]leucine (74 GBq) and unlabeled L-leucine (25 μmol/L) in a total of 5 mL. The medium was completely removed and replaced with fresh medium with and without HDL_3 but containing no FBS, and the incubations were continued. At the end of 1.5, 3.0, 4.5, and 6 h, the incubation medium from one set each of the nonstimulated and stimulated control and alcohol groups was transferred into ice-cold tubes and centrifuged at 500 × *g* for 5 min at 4°C. After centrifugation, the cells were washed 3 times with ice-cold 10 mmol PBS/L. The medium collected from each reaction sample was analyzed individually for incorporation of labeled leucine into immunoprecipitable apo E according to our published method (6). A known volume of the pooled medium (secreted apo E fraction) and the whole cell homogenate (3%, wt:vol, intracellular apo E fraction) was passed through an anti-rat apo E Sepharose 4B column to purify apo E as described below. The authenticity

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of the eluted pure apo E from the column was checked by Ouchterlony analysis. Pure apo E fractions, obtained thus from the intracellular and secretory fractions of the control and alcohol groups, were processed for enzymatic hydrolysis by using exo-α-sialidase (neuraminidase; EC 3.2.1.18; 14). Finally, the sialic acid content of the apo E fractions was determined according to the published protocol of Warren (15).

Experiment 3: effects of chronic alcohol intake on the binding characteristics of secreted apo E to HDL_3 in vitro

Five hundred micrograms each of the pure apo E fractions obtained from the secreted medium of the control and alcohol-treated groups (experiment 2) was delipidated (chloroform: methanol, 1:1, by vol) and processed for complete desialylation by using neuraminidase as described below. Additionally, known amounts of the purified, intracellular and secreted apo E fractions from the control and alcohol-treated groups (500 μg) were processed for the resialylation reaction by using β-galactosamide α-2,6-sialyltransferase (sialyltransferase; EC 2.4.99.1) as described below. The sialic acid content was determined in these apo E fractions after enzymatic hydrolysis. Furthermore, the unmodified, desialylated, and resialylated apo E fractions of the control and alcohol-treated groups were labeled with ^{125}I according to the method of Bolton and Hunter (16). Thus, the labeled apo E (146 200 cpm/mg protein) of the control compared with the alcohol-treated rats was found to have molar ratios of sialic acid to protein of 0/0, 0.96/1.93, and 1.90/1.90, respectively, in its deglycosylated, unmodified, and reglycosylated form. The purity and authenticity of labeled unmodified, desialylated, and resialylated apo E were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Ouchterlony analysis and subjected to binding studies.

Desialylation and resialylation of secreted apo E

The enzymes used in these reactions have repeatedly shown no proteolytic activity. Both the desialylation and resialylation incubations were carried out at 37°C for 4 h in 0.5 mL of 100 mmol cacodylate buffer/L (pH 6.0) containing 500 μg apo E protein and 10 μg of either neuraminidase (desialylation reaction) or sialyltransferase (resialylation reaction). After the reaction, the content was passed through an anti-apo E affinity column to recover pure apo E. The purity of apo E was checked by SDS-PAGE. Aliquots of pure apo E were processed for protein analysis and determination of sialic acid in all fractions.

Preparation of the apo E–lipid complex

For the preparation of the apo E–lipid complex, the published protocol of Milla and Hirschberg (17) was followed, with slight modifications. Briefly, egg yolk phosphatidylcholine (1500 μmol; Sigma Chemical Co) and cholesterol (500 μmol; Sigma Chemical Co) in hexane were dried on the walls of a 10-mL glass ampoule with nitrogen gas and lyophilized overnight. The dried lipid was resuspended in 1 mL dimethylsulfoxide to obtain a clear solution. Dialyzed samples of unmodified, desialylated, and resialylated labeled apo E (200 μmol in 0.1 mL) in 200 mmol phosphate buffer/L, pH 7.4, isolated from the secreted medium of the alcohol and control macrophages, were individually mixed with lipids in dimethylsulfoxide by gentle stirring. The mixtures were then quickly frozen in a dry ice–acetone bath and allowed to thaw at room temperature (15 min). The cycle was repeated 5 times. After the fifth thawing, the mixtures were sonicated for 10 s, applied to a Sephadex G-15 column, and eluted with phosphate buffer (20 mmol/L, pH 7.4). Fractions of 2.0 mL were collected. An aliquot of the fractionated apo E–lipid complex was characterized by SDS-PAGE and its protein, sialic acid, and lipid contents were determined. The apo E–lipid complex fraction was eluted in the void volume and was used for binding studies.

Experiment 1: binding of labeled apo E–lipid complex to HDL_3

Rat plasma HDL_3 fractions were isolated from control rats and characterized for their protein and lipid contents. Triplicate samples of unmodified, desialylated, and resialylated labeled apo E–lipid complex (200 μg protein) isolated from the control and alcohol-treated groups were added individually to 5 sets of HDL_3 fractions (500 μg protein) and incubated at 37°C. The reaction was carried out for 15, 30, 60, 90, and 120 min. The reaction mixture was subjected to dextran sulfate precipitation for HDL_2 isolation according to the method described by Gavish et al (18). The isolated HDL_2 precipitate was washed twice with saline solution and dissolved in sodium citrate buffer (200 mmol/L, pH 8.3). An aliquot was taken for SDS-PAGE and lipid analysis to verify the purity and authenticity of the HDL_2 subfraction. The solution containing HDL_2 was dialyzed against saline solution for 24 h and reprecipitated with dextran sulfate. The precipitate was vacuum-dried and the radioactivity of bound [^{125}I]apo E in HDL was determined in an AutoGamma 5000 series spectrometer (Packard Instruments, Downers Grove, IL). The results are expressed as nmol bound apo E/μmol HDL.

Statistical analysis

All the data were analyzed to evaluate the interaction between alcohol and HDL stimulation; two-factor analysis of variance (ANOVA) was used, and unless there was a significant interaction between the 2, only the main effects are presented. Wherever appropriate, the results obtained for the control and alcohol groups were compared by using Student's two-tailed *t* test (19).

RESULTS

Uptake of acetylated LDL by alcohol-treated and control rat peritoneal macrophages

When freshly isolated peritoneal macrophages from control and alcohol-treated rats were incubated with increasing concentrations of acetylated LDL, the cellular uptake of LDL cholesterol was linear throughout the 6 h of incubation in both groups. Although the uptake rate by alcohol-treated macrophages was 20% slower ($P < 0.05$) than that of the control macrophages (**Figure 1**), the cholesterol concentration in the peritoneal macrophages from alcohol-treated rats was 33.3% higher ($P < 0.05$) than that in macrophages from control rats after 24 h of incubation.

Subcellular fractionation

The purity and yield of microsomes and Golgi fractions isolated from macrophages were assessed by measurement of their marker enzyme activities. Accordingly, we found that 87% of NADPH–cytochrome *c*2 reductase (EC 1.6.2.5) activity was associated with the microsomal fraction with very little contamination by galactosyltransferase. Similarly, 91% galactosyltransferase activity was found in the Golgi fraction with very little contamination by NADPH–cytochrome *c*2 reductase activity.

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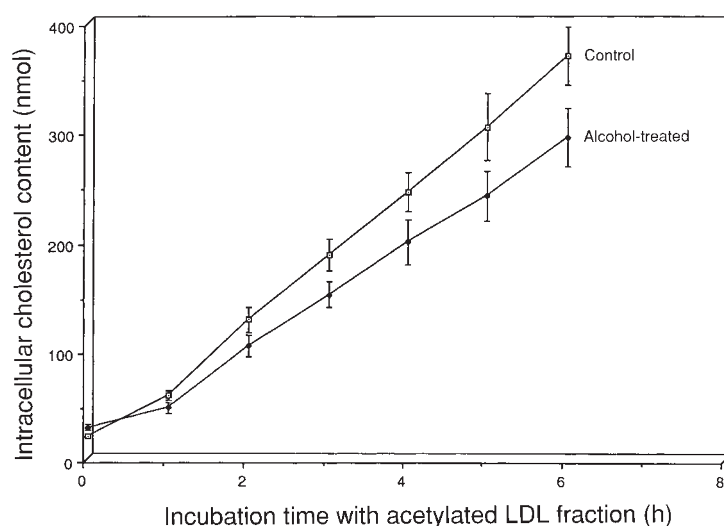

FIGURE 1. Mean (±SE) cholesterol content of 6 separate peritoneal macrophage colonies isolated, respectively, from the 6 rats in each group. Peritoneal macrophages were isolated from rats fed control and alcohol liquid diets for 8 wk. Cells were plated and isolated as described in Methods. Six sets (6×10^6 adherent cells/dish) of these isolated macrophages from control and alcohol groups were then incubated with acetylated LDL and followed up to 24 h in a medium containing 10% fetal bovine serum. The cholesterol-laden macrophages were then processed for their lipid fractionation, and cholesterol content was determined. The rate of LDL uptake by alcohol-treated macrophages was significantly different from that of the control macrophages, $P < 0.05$.

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Effect of chronic alcohol treatment on protein content of rat peritoneal macrophages

The protein content of macrophages was 21.4% greater in the whole cells and 29.2% greater in the Golgi fractions from the alcohol-treated group than in those from the control group, whereas there was no significant difference in protein contents at the microsomal level (**Table 1**).

Effect of chronic alcohol treatment on the relative synthetic rate of apo E and its relative sialylation rate in nonstimulated and stimulated rat peritoneal macrophages

The incorporation of labeled leucine into total protein (trichloroacetic acid precipitable) and immunoprecipitable apo E and the incorporation of labeled ManNAc into immunoprecipitable apo E in whole cells, microsomes, and in the Golgi fractions of peritoneal macrophages of the control and alcohol-treated groups under stimulated and nonstimulated conditions were determined. To nullify any variations between the groups in the intracellular pool size of labeled precursors, the results are expressed in **Table 2** as the apo E synthetic rate relative to the total protein synthetic rate (relative apo E synthetic rate) and as the apo E sialylation rate relative to its protein synthetic rate (relative apo E sialylation rate). Two-factor ANOVA of the data showed that there was no interactive effect of alcohol and HDL on the relative synthetic and sialylation rates of apo E. Chronic alcohol treatment did not significantly affect the relative synthetic rate of apo E in the macrophages or in any of their subcellular compartments regardless of whether the macrophages were nonstimulated or stimulated by HDL (Table 2). In contrast, it is clear in Table 2 that, compared with the control group, the alcohol-treated group had a significant, 51–57% lower relative rate of sialylation of apo E at the whole-cell and Golgi levels, regardless of whether the macrophages were stimulated by HDL.

Kinetics of apo E secretion by nonstimulated and stimulated rat peritoneal macrophages pretreated with and without alcohol

The kinetic patterns of newly synthesized apo E secretion by rat peritoneal macrophages in nonstimulated and stimulated conditions are presented in **Figure 2**. Two-factor ANOVA of the data showed no interaction between alcohol and HDL stimulation on this variable. Stimulation of cells by HDL resulted in a 74.8–196.0% increase ($P < 0.001$) in the rate of secretion of newly synthesized apo E from rats of the control group and a 136.4–305.5% increase ($P < 0.001$) in that from rats of the alcohol-treated group compared with their nonstimulated counterparts (Figure 2). Chronic alcohol consumption markedly impaired the secretion of newly synthesized apo E by macrophages, irrespective of the presence or absence of HDL in the medium ($P < 0.001$).

TABLE 1
Effects of chronic alcohol treatment on protein content of rat peritoneal macrophages and subcellular organelles[1]

Treatment group	Protein		
	Whole cell	Microsomes	Golgi apparatus
		mg/g cell wt	
Control	11.2 ± 0.8	5.7 ± 0.4	2.4 ± 0.1
Alcohol-treated	13.6 ± 0.7[2]	6.5 ± 0.4	3.1 ± 0.1[2]

[1] $\bar{x}$ ± SE; $n = 6$ separate peritoneal macrophage colonies isolated from 6 animals. Peritoneal macrophages were isolated from rats fed control and alcohol liquid diets for 8 wk. The isolated macrophages of the control and alcohol-treated groups were homogenized (3%, wt:vol) in 0.5 mol sucrose/L made in sodium phosphate buffer (0.2 mol/L, pH 7.4). Microsomes and Golgi apparatus were isolated from the cell homogenate by ultracentrifugation using a discontinuous sucrose gradient. The protein content of each fraction was determined as described in Methods.

[2] Significantly different from the control group, $P < 0.05$.

TABLE 2
Effects of chronic alcohol treatment on the relative apolipoprotein (apo) E synthetic rate and relative sialylation rate of apo E in peritoneal macrophages[1]

	Relative apo E synthetic rate[2]		Relative apo E sialylation rate[3]	
Condition	Control	Alcohol-treated	Control	Alcohol-treated
Nonstimulated				
Total cells	0.068 ± 0.003	0.076 ± 0.006	0.30 ± 0.02	0.14 ± 0.01[4]
Microsomes	0.043 ± 0.002	0.052 ± 0.003		
Golgi apparatus	0.089 ± 0.007	0.120 ± 0.010	0.82 ± 0.08	0.39 ± 0.02[4]
Stimulated				
Total cells	0.078 ± 0.006	0.090 ± 0.008	0.37 ± 0.02	0.16 ± 0.01[4]
Microsomes	0.052 ± 0.003	0.054 ± 0.003		
Golgi apparatus	0.120 ± 0.006	0.130 ± 0.009	0.86 ± 0.08	0.42 ± 0.03[4]

[1] $\bar{x}$ ± SE; n = 6 peritoneal macrophage cultures isolated from 6 rats. Peritoneal macrophages were isolated from rats fed control and alcohol liquid diets for 8 wk and cultured as described in Methods (≈6×10^6 adherent cells/dish in ≈72 h). The isolated macrophages were then incubated with acetylated LDL for 24 h in a medium containing 10% fetal bovine serum, resulting in an accumulation of cholesterol. The cholesterol-laden macrophages were then maintained in a medium free of fetal bovine serum in the presence of 500 μg HDL. Labeled leucine and labeled *N*-acetyl-D-mannosamine (ManNAc) were added to the medium and incubated for 60 min as described in Methods. After incubation, the cells were processed for isolation of microsomes and Golgi apparatuses by ultracentrifugation using a discontinuous sucrose gradient. Radioactivity of the labeled leucine and ManNAc incorporated into total protein [trichloroacetic acid (TCA) precipitable] and apo E (immunoprecipitable) were determined as described in Methods. Each immunoprecipitable value was corrected for nonspecific precipitation that was <5%. Two-factor ANOVA revealed that there was no significant effect of alcohol or HDL stimulation nor a significant interaction between alcohol and HDL stimulation with respect to apo E synthetic rate.

[2] Ratio of incorporation of labeled leucine into immunoprecipitable apo E to that into TCA-precipitable total protein.

[3] Ratio of incorporation of labeled ManNAc to labeled leucine into immunoprecipitable apo E; because there was no incorporation of ManNAc, no values are reported for the microsomes.

[4] Significant main effect of alcohol, $P < 0.001$, on the relative sialylation rate of apo E (but there was no significant interaction between alcohol and HDL stimulation with respect to this variable).

Sialic acid content of intracellular and secreted apo E of rat peritoneal macrophages with and without alcohol pretreatment

Our results showed that the sialic acid residues of intracellular apo E synthesized by rat peritoneal macrophages that had undergone pretreatment with chronic alcohol were significantly lower than those synthesized by macrophages isolated from control rats. Similarly, a significantly lower molar ratio of sialic acid in secreted apo E was found in the case of those secreted by rat peritoneal macrophages pretreated with alcohol than in those secreted by control macrophages (**Table 3**).

Effect of chronic alcohol treatment on the HDL binding properties of apo E secreted by rat peritoneal macrophages: effects of sialylation and desialylation of apo E

The relative binding capacities of newly synthesized and secreted apo E isolated from control and alcohol-treated rats to HDL_3 are compared in **Figure 3**. The characteristic binding curve showed a progressive increase in the binding of apo E from the control and alcohol groups to HDL_3 from 15 to 90 min, after which it reached a plateau. The HDL_3 binding ability of apo E from the alcohol-treated rats at 90 min of incubation was 36.6% lower than that of the control group (Figure 3).

The effects of desialylation and resialylation of apo E on the extent of its binding to HDL as a function of time is shown in **Figure 4**. Here again, there was no interaction between alcohol and the degree of sialylation of apo E on binding. Significantly, compared with the binding capacity of the corresponding native apo E from each group, complete removal of sialic acid from apo E resulted in loss of 27.6–52.6% of its binding capacity to HDL_3 in the alcohol-treated group as opposed to 47.65–67.25% loss in the control group. More importantly, resialylation of the desialylated species of apo E secreted by macrophages from both the control and alcohol-treated groups resulted in complete recovery of the ability to bind to the HDL_3 molecule (Figure 4), similar to the binding ability of apo E from the control group shown in Figure 3.

DISCUSSION

First, the process of reverse cholesterol transport needs to be described so that the effects of alcohol on HDL function can be fully understood. Liver and macrophages are likely to be the major sites of production of apo E, which is bound to plasma lipoproteins after secretion (20–22). Newly synthesized apo E emerges bound to phospholipids in the form of lamellar discs. In addition to apo E and phospholipids, these discs contain apo A-I and are referred to as nascent HDL particles. Once in circulation, the native HDL picks up cholesterol from peripheral tissues such as macrophages and is converted into HDL_3. The apo A-I component of HDL_3 activates phosphatidylcholine–sterol *O*-acyltransferase (lecithin-cholesterol acyltransferase; LCAT), which esterifies the free cholesterol component. Thus, the lipid core of HDL_3 is expanded, leading to the formation of less dense HDL_2 (20–22). Cholesterol-loaded macrophages have been identified as another potential source of apo E (22). Thus, mouse and human macrophages accumulated a large amount of cholesterol when incubated with modified LDL such as acetylated or malondialdehyde-treated LDL (23, 24). Within the cell, the cholesterol esters are hydrolyzed in lysosomes and the free cholesterol is secreted into the medium when the culture medium contains HDL (25, 26). The apo E secreted by both the liver and macrophages is highly sialylated (27, 28). Presumably, the phospholipid fatty acids of apo E–phospholipid discs secreted by the cell serve as substrates for LCAT to esterify the free cholesterol picked up by HDL. This process leads to the formation of cholesterol ester–rich HDL (HDL_C). In the final step, cholesterol

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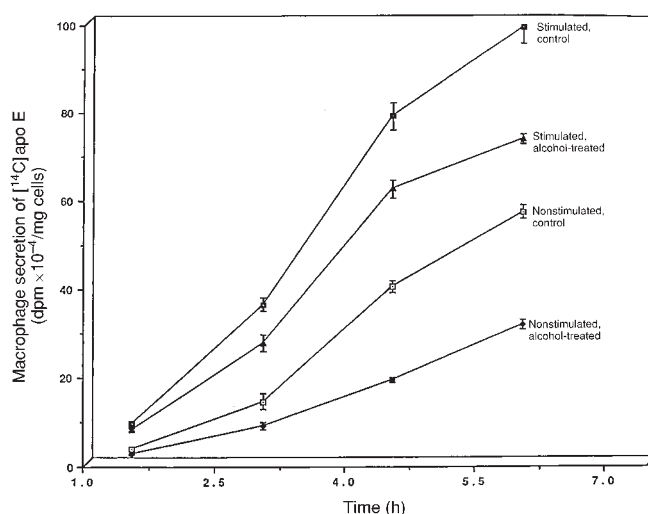

FIGURE 2. Mean (±SE) binding of labeled apolipoprotein (apo) E to HDL in 6 separate peritoneal macrophage colonies isolated, respectively, from 6 rats in each group. Peritoneal macrophages were isolated from control and alcohol-treated rats and cultured as described in Figure 1 and Methods. The isolated macrophages were then incubated with acetylated LDL for 24 h in a medium containing 10% fetal bovine serum, resulting in an accumulation of cholesterol. The cholesterol-laden macrophages were then maintained in a medium free of fetal bovine serum in the absence and presence of HDL. Labeled leucine was added to the medium (for 60 min), then the medium was replaced with no labeled leucine. The secretion of labeled apo E was then followed up to 6 h as described in Methods. Apo E in the secreted medium was immunoprecipitated and radioactivity was determined. Values were adjusted for nonspecific precipitation (<5%). There was no significant difference between treatment groups in the effects of HDL stimulation.

ester–rich HDL (which may be either HDL_2 or HDL_C) is delivered to the liver via the apo E receptor–mediated process for degradation. This whole process is reverse cholesterol transport, in which HDL plays a major role.

In agreement with previous reports (29, 30), peritoneal macrophages of both the alcohol-treated and the control rats showed increased uptake and increased content of cellular cholesterol when incubated with acetylated LDL (Figure 1). However, we found that macrophages from chronically alcohol-fed rats had a lower rate of LDL-cholesterol uptake than did those isolated from control rats. This may be attributed to a possible alcohol-mediated reduction in the number of receptor binding sites or alterations in the binding affinity of these receptors for acetylated LDL. These aspects remain to be determined. Accumulation of 33% more cholesterol after 24 h of incubation in macrophages from the alcohol group than from the control group could be attributed to lower efflux rate of cellular cholesterol in the alcohol group than in the control group.

Plasma apo E is an O-type sialoglycoprotein (7, 31). The liver appears to be the main site of apo E synthesis, although most peripheral tissues, including macrophages, can synthesize apo E (6). The mechanism and involvement of subcellular organelles, particularly the Golgi apparatus and microsomes, in the glycosylation process were reviewed previously (32). Chronic alcohol treatment markedly inhibited the relative sialylation rate of apo E synthesized in rat peritoneal macrophages without affecting the relative apo E protein synthetic rate (Table 2). This defect in apo E sialylation may have been a result of damaged glycosylation machinery in peritoneal macrophages of rats chronically pretreated with alcohol. Chronic alcohol treatment of rats might have severely affected the activities of sialyltransferase, neuraminidase, or both. We showed earlier (5) that chronic alcohol treatment in rats has deleterious effects on these 2 enzymes in rat liver and impairs both glycosylation and secretion of apo E from the liver (5, 8).

Our pulse-chase secretion kinetics studies showed that the presence of HDL_3 accelerates apo E secretion in cholesterol-laden macrophages (Figure 2). This agrees with the results of studies reported previously by Dory (33) and Brown and Goldstein's group (6). The diminished rate of apo E secretion by macrophages chronically pretreated with alcohol may be attrib-

TABLE 3
Effect of chronic alcohol consumption on sialic acid content of intracellular and secreted apolipoprotein (apo) E by rat peritoneal macrophages[1]

Fraction and treatment group	Sialic acid	Molar ratio (sialic acid:apo E)	Percentage decrease
	nmol/mg protein		*%*
Intracellular apo E			
Control	42.92 ± 6.32	1.46	—
Alcohol-treated	24.99 ± 1.98[2]	0.85[2]	41.78
Secreted apo E			
Control	56.84 ± 4.98	1.93	—
Alcohol-treated	28.36 ± 2.03[2]	0.96[2]	50.26

[1] Peritoneal macrophages were isolated from rats fed control and alcohol liquid diets for 8 wk and cultured as described in Methods. The isolated macrophages were then incubated with acetylated LDL for 24 h in a medium containing 10% fetal bovine serum, resulting in an accumulation of cholesterol. The cholesterol-laden macrophages were then maintained in a medium free of fetal bovine serum, in the absence and presence of HDL. The macrophages and the medium were collected and removed after 6 h. Intracellular and secreted apo E were purified, respectively, from the macrophage homogenate and medium by using an anti-rat apo E–Sepharose 4B immunoaffinity column. The pure apo E was characterized by electrophoresis. The pure protein was acid hydrolyzed. The liberated sialic acid was determined as described in Methods.

[2] Significantly different from the control value, $P < 0.001$.

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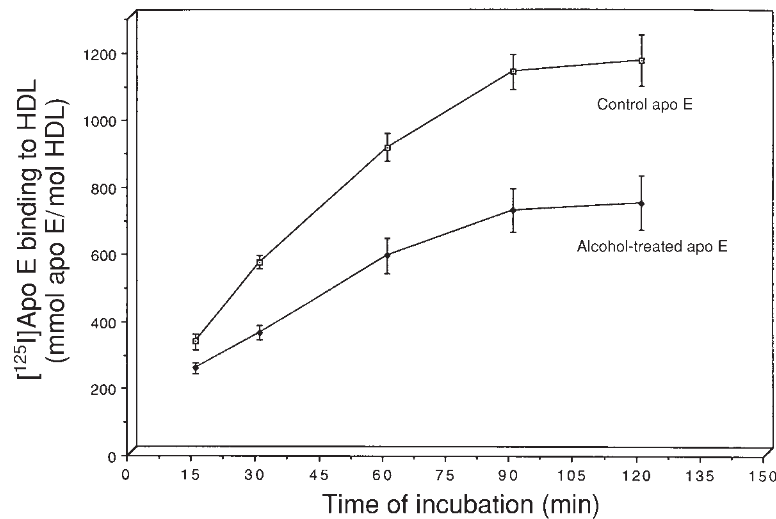

FIGURE 3. Mean (±SE) binding of labeled apolipoprotein (apo) E to HDL_3 in 6 separate peritoneal macrophage colonies isolated, respectively, from 6 rats in each group. Peritoneal macrophages were isolated from control and alcohol-treated rats and cultured as described in Figure 1 and Methods. These cultures were maintained in medium in the presence of 500 μg HDL_3 but no fetal bovine serum. The medium was collected after 6 h of incubation and the secreted apo E was purified by using an anti-rat apo E Sepharose 4B immunoaffinity column (Pharmacia, Uppsala, Sweden), and then characterized by electrophoresis. The pure protein was iodinated as described in Methods and iodinated apo E was then incubated with HDL_3 fractions isolated from control rats. After incubation, the HDL_2 fraction was isolated from the medium and the radioactivity of the bound labeled apo E in the HDL_2 fraction was determined as described in Methods. HDL_3 binding ability of apo E from alcohol-treated rats was significantly different from that of apo E from control rats, $P < 0.001$.

uted to alcohol-mediated defective sialylation of apo E by macrophages, leading to alterations in its conformation. Another possibility could be an alcohol-induced, defective response of the macrophages to HDL_3 stimulation. It has been suggested that stimulation of apo E secretion by macrophages is mediated by an HDL-receptor interaction. Chronic exposure to alcohol might impair the receptor interaction and lead to diminished apo E secretion. In either case, the functional implications

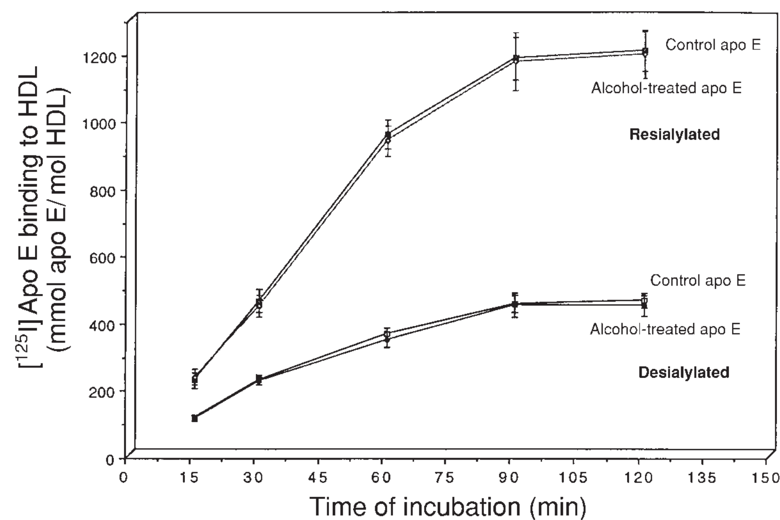

FIGURE 4. Mean (±SE) binding of labeled apolipoprotein (apo) E to HDL_2 in peritoneal macrophage colonies isolated from control and alcohol-treated groups of rats and cultured as described in Figure 1 and Methods, in the presence of 500 μg HDL but no fetal bovine serum. The medium was collected after 6 h of incubation and the secreted apo E was purified as described in Figure 3. The purified apo E was further treated with neuraminidase (exo-α-sialidase; EC 3.2.1.18) or sialyltransferase (β-galactosamide α-2,6-sialyltranferase; EC 2.4.99.1) to completely desialylate or resialylate the protein. The modified and reconstituted apo E was characterized by electrophoresis. The pure protein was iodinated as described in Methods and the iodinated desialylated or resialylated apo E was then incubated with HDL_3 fractions isolated from rats in the control group. After a specific incubation, the HDL_2 fraction was isolated from the medium and the radioactivity of the bound labeled apo E in HDL_2 fraction was determined as described in methods. The HDL_3 binding capacity of apo E from the alcohol-treated group was reduced by 27.6–52.6% with complete removal of the sialic acid residues ($P < 0.001$) and that of the apo E from the control group was reduced by 47.65–67.25% ($P < 0.001$).

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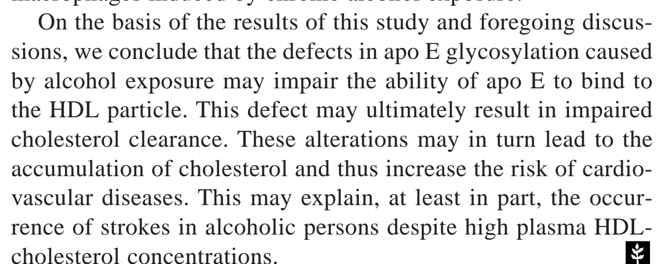
of this defect would ultimately be nonavailability of apo E to secreted cholesterol for its clearance.

The glycosylation machinery in the macrophage system has not been delineated. Whether sialylation of apo E is necessary for the secretion of apo E by macrophages remains to be explored further. A high degree of sialylation in secretory apo E may be essential for its stable molecular conformation and attachment to phospholipid discs. Sialic acid residues of apo E may also play a role in its recognition by the HDL_3 molecule. This would explain the loss of binding affinity of desialylated apo E to HDL_3. It is only after HDL_3 incorporates apo E along with secreted cholesterol that it transforms into HDL_2, a form that is recognized by the liver for further degradation. Integration of newly secreted apo E into the HDL_3 molecule is a physiologic process that converts HDL_3 into HDL_2, which is then taken up by the liver for cholesterol degradation. Sialic acid residues of secreted apo E may play a crucial role in the integration of apo E into HDL_3 or its recognition by surface receptors in the liver. We speculate that a specific molecular conformation of apo E may be necessary for both integration of apo E into the HDL particle and its recognition by surface receptors. Sialic acid residues may impart a specific conformation to the apo E molecule regardless of whether it is free apo E or in the bound form within the HDL particle. More efficient and specific recognition of apo E may be dependent on a particular conformation of apo E that is determined by its sialic acid content.

The results of this study suggest that depletion of sialic acid residues from newly secreted apo E, caused by chronic exposure of macrophages to alcohol, resulted in the partial inability of apo E to bind to the HDL_3 molecule. This is supported by our findings that complete depletion of sialic acid residues from secreted apo E from both control and alcohol-pretreated macrophages dramatically impaired apo E integration into the HDL particle (Figure 4). Furthermore, resialylation of apo E could completely restore the ability of apo E to integrate within the HDL molecule (Figure 4). It is intriguing to find that resialylation of apo E secreted even by alcohol-pretreated macrophages resulted in complete restoration of the ability of apo E to integrate within the HDL particle. We therefore logically conclude that sialic acid residues are absolutely necessary to provide a specific and final molecular conformation to apo E so that it can integrate within the HDL particle. Further studies are necessary to examine the functional implications of this defect in apo E glycosylation in macrophages induced by chronic alcohol exposure.



On the basis of the results of this study and foregoing discussions, we conclude that the defects in apo E glycosylation caused by alcohol exposure may impair the ability of apo E to bind to the HDL particle. This defect may ultimately result in impaired cholesterol clearance. These alterations may in turn lead to the accumulation of cholesterol and thus increase the risk of cardiovascular diseases. This may explain, at least in part, the occurrence of strokes in alcoholic persons despite high plasma HDL-cholesterol concentrations.

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