Cholesterol Efflux to High-Density Lipoproteins and Apolipoprotein A-I Phosphatidylcholine Complexes Is Inhibited by Ethanol: Role of Apolipoprotein Structure and Cooperative Interaction of Phosphatidylcholine and Cholesterol†

Nicolai A. Avdulov, Svetlana V. Chochina, Urule Igbavboa, and W. Gibson Wood*

Geriatric Research, Education and Clinical Center, VA Medical Center, and Department of Pharmacology, University of Minnesota School of Medicine, Minneapolis, Minnesota 55417

Received April 14, 2000; Revised Manuscript Received June 30, 2000

ABSTRACT: There is a substantial body of evidence showing that moderate alcohol consumption is associated with a reduced risk of cardiovascular morbidity and mortality. One of the factors thought to contribute to this reduction in risk is an increase in the level of high-density lipoproteins (HDL) correlated with alcohol consumption. However, HDL levels are elevated in heavy drinkers, but their risk of vascular disease is greater compared with that of moderate drinkers. Ethanol at concentrations observed in heavy drinkers and alcoholics may directly act on HDL and apolipoproteins and in turn modify cholesterol efflux. In this paper, we show that ethanol significantly inhibited cholesterol efflux from fibroblasts to HDL and to apolipoprotein A-I (apoA-I) complexed with phosphatidylcholine (PC). Ethanol significantly inhibited binding of PC to apoA-I, inhibited incorporation of cholesterol only when apoA-I contained PC, and did not alter incorporation of cholesterol into HDL. ApoA-I structure was altered by ethanol as monitored by steady-state fluorescence polarization of tryptophan residues. The absence of ethanol effects on incorporation of cholesterol into HDL versus inhibition of cholesterol incorporation into the apoA-I−PC complex suggests that the effects of ethanol on cholesterol efflux mediated by HDL involve interaction with the cell surface and that efflux mediated by the apoA-I−PC complex is a combination of aqueous diffusion and contact with the cell surface. In addition, effects of ethanol on apoA-I suggest that pre-β-HDL or lipid-free apoA-I may be more perturbed by ethanol than mature HDL, and such effects may be pathophysiological with respect to the process of reverse cholesterol transport in heavy drinkers and alcoholics.

Moderate alcohol consumption reduces the risk of cardiovascular disease morbidity and mortality, whereas heavy alcohol consumption increases that risk (*1*−*3*). The reduced risk attributed to moderate alcohol consumption has been proposed to be due in part to an increase in the level of high-density lipoproteins (HDL).[1] A generally accepted finding of both epidemiological and experimental studies is that levels of HDL are increased in association with alcohol consumption (*4*−*9*). HDL play an important role in reverse cholesterol transport (*10*−*14*), and an increase in levels of HDL associated with moderate alcohol consumption may facilitate removal of cholesterol from cells and transport to the liver. However, levels of HDL are increased in heavy drinkers, but their risk of cardiovascular disease is higher than that of moderate alcohol drinkers (*6*, *8*, *9*).

The molecular mechanisms that are involved in the interaction between HDL and alcohol consumption are not well-understood (*5*−*9*). Moreover, little is known concerning actions of ethanol on HDL and cholesterol efflux from cells. Cholesterol is removed from cells by HDL and lipid-free and lipid-poor apolipoproteins (*10*−*13*). A very high concentration of ethanol (350 mM), approximately 16 times higher than what is defined as legal intoxication, was reported to increase the rate of cholesterol efflux from red blood cells to plasma (*15*). While effects of ethanol in that study have to be viewed cautiously, it would appear that ethanol can affect cholesterol efflux. Mechanisms of effects of ethanol on cholesterol efflux could involve physicochemical interactions with HDL and lipid-poor and lipid-free apolipoproteins. Ethanol was shown to directly bind to BSA using NMR and fluorescence spectroscopy (*16*). Binding of lipids to BSA and sterol carrier protein-2 (SCP-2) was inhibited by ethanol concentrations (beginning at 25 mM) typically observed in heavy drinkers (*16*, *17*). Experiments reported in this paper were designed to examine effects and potential mechanisms of actions of ethanol on cholesterol efflux at ethanol concentrations commonly observed in heavy drinkers. The following hypotheses were tested in a series of experiments using cultured fibroblasts, dehydroergosterol (DHE), 22-(*N*-

† This work was supported by grants from the National Institutes of Health (AA-10806) and the Department of Veterans Affairs.

* To whom correspondence should be addressed: VA Medical Center, GRECC 11G, One Veterans Drive, Minneapolis, MN 55417. Phone: (612) 725-2051. Fax: (612) 725-2084. E-mail: Woodx002@tc.umn.edu.

[1] Abbreviations: HDL, high-density lipoproteins; apoA-I, apolipoprotein A-I; PC, phosphatidylcholine; BSA, bovine serum albumin; SCP-2, sterol carrier protein-2; DHE, dehydroergosterol; NBD-cholesterol, 22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol; NBD-PC, 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-43-yl)amino]dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; DMF, dimethylformamide; DPH, 1,6-diphenyl-1,3,5-hexatriene; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DMEM, Dulbecco's modified Eagle's medium; CREF, cloned rat embryo fibroblasts; FBS, fetal bovine serum; apoE4, apolipoprotein E4; SR-BI, scavenger receptor class B type I; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; ABC1, ATP-binding cassette transporter 1; SEM, standard error of the mean.

10.1021/bi0008534 CCC: $19.00
Published on Web 08/02/2000

7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol (NBD-cholesterol), and 22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol (NBD-PC). We determined if ethanol would modify cholesterol efflux mediated by HDL, lipid-free apoA-I, or apoA-I complexed with PC. Ethanol-induced effects on HDL-mediated cholesterol efflux could result from ethanol modifying the incorporation of cholesterol into HDL, and this potential mechanism was examined. It has been previously proposed that effective cholesterol efflux may require phospholipids associated with the apolipoprotein (*10*, *11*). Cholesterol efflux was enhanced by apoA-I containing PC (*12*). Ethanol could alter binding of PC to apoA-I; such an effect could modify interaction of the apoA-I−PC complex with cholesterol, and this potential mechanism was examined. Modification of apolipoprotein structure can alter lipid binding capacity and lipid efflux (*11*, *14*, *18*). Apolipoprotein structure could be altered by ethanol, and this hypothesis was tested using fluorescence polarization of apoA-I tryptophan residues, a method that has been previously shown to reveal changes in apoA-I structure (*19*).

EXPERIMENTAL PROCEDURES

Materials

Apolipoproteins A-I and E4 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). 22-(*N*-7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol (NBD-cholesterol), 22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol (NBD-PC), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes (Eugene, OR). Dehydroergosterol (DHE), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, human plasma HDL, and all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Cell Culture. Cloned rat embryo fibroblasts (CREF) were a kind gift from V. Polunovskyi. Cells were maintained in DMEM containing 10% FBS and 2 mM L-glutamine at 37 °C in a humidified incubator with 5% CO_2. Fibroblasts were grown as monolayers in 50 mL flasks or 60 mm plastic Petri dishes, at a density of 1×10^6 cells per flask (5 mL of DMEM) for 2 days. All experiments were performed with confluent cells. Cell viability was determined by lactate dehydrogenase (LDH) activity. Fibroblasts (after growing for 2 days) were incubated with 3 μg/mL DHE for 24 h, and then the cells were washed twice with DHE-free medium. A 24 h time interval for incubation was chosen because in a separate set of experiments we found that DHE fluorescence intensity in cells reaches saturation at approximately 24 h. DHE fluorescence intensity is proportional to the amount of DHE in the fibroblasts. The amount of DHE was determined in cells trypsinized, collected, and centrifuged at 14 000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice in 1 mL of PBS and homogenized in a loose-fitting Teflon homogenizer. Quantification of DHE was carried out by HPLC using procedures we and others have previously reported (*20*−*22*). DHE was analyzed on an HPLC Adsorbosil C_{18} column (diameter, 5 μm; length, 250 mm; Alltech Associates Inc.). The solvent system was HPLC-grade acetonitrile and methanol (30:70) at a flow rate of 1 mL/min with a Rainin Dynamax model SD-200 solvent delivery system (Rainin Instrument Co. Inc.). The column eluant was monitored for absorbance at 215 nm using a Rainin Dynamax model PDA-1 photodiode array detector. The standard curve for DHE was generated by injecting different concentrations of DHE onto the column. DHE was quantified using a Rainin Dynamax PC HPLC data sytem.

ApoA-I−*PC Complexes*. POPC in chloroform (3.2 mg) was dried under nitrogen. Tris/saline (1.0 mL, pH 8.0) was then added to the tubes, and samples were vortexed vigorously for 3 min to resuspend the POPC. The mixture was sonicated for 1 min at 100% duty cycle using a Branson Sonifier 250 sonicator with a $^1/_8$ in. tapered microtip probe and an output control setting at 3 (manufacturer-rated output of 40 W). Samples were then incubated in a water bath at 37 °C for 30 min and sonicated again at 95% duty cycle for 5 min. All sonications were performed in 12 mm × 75 mm test tubes in a 15 °C water bath and under nitrogen. ApoA-I (2 mg of a 1.4 mg of protein/mL buffer) was added to the tube, and samples were sonicated again for 4 × 1 min at 90% duty cycle with a 1 min cooling period between each sonication. The resulting mixtures were passed through a 0.22 μm syringe tip filter, and then dialyzed over 2 days, against four changes of buffer 2 L each in a 2.0 mL dialyzer [Z36, 840-7, MWCO of 50 000 Da, Sigma] to remove free lipids and protein.

Fluorescence Spectroscopy. A LS-50B fluorimeter (Perkin-Elmer, Norwalk, CT) was used for the fluorescence measurements. Band-pass slits of 10 and 5 nm were used for fluorescence excitation and emission, respectively. For measurements using DHE, the excitation wavelength was set at 318 nm and the emission wavelength at 380 nm. For NBD-labeled lipids, an excitation wavelength of 460 nm was used with emission being recorded at 530 nm. Fluorescence intensity was corrected for light scatter by using samples identical to the other samples but not containing DHE or fluorescent labeled lipids and subtracting background fluorescence. Steady-state polarization of DPH was assessed in cells as an indicator of fluidity using procedures that we have reported previously from our laboratory (*20*, *21*, *23*, *24*). Steady-state fluorescence polarization of the tryptophan residues in apoA-I was assessed using a wavelength of 280 nm. The cuvette temperature in all experiments was maintained at 36.5 ± 0.1 °C in a thermostated chamber using a circulating water bath and continuous stirring.

Cholesterol Efflux. Confluent fibroblasts preloaded with DHE for 24 h were incubated with different concentrations of HDL (5−40 μg of protein/mL of cell culture medium) for 2 h to establish an optimal HDL concentration. HDL (30 μg of protein/mL of cell culture medium) was used in the cholesterol efflux experiments in which the effects of ethanol were examined. Cells were incubated for 30 min with 25 or 50 mM ethanol or no ethanol, after which 30 μg of human plasma HDL protein/mL of medium or 20 μg of the apoA-I−PC complex was added and cells were incubated for 2 h. Cells were then washed twice with medium that did not contain HDL or the apoA-I−PC complex, trypsinized, collected, and centrifuged at 14 000 rpm for 1 min in an

Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice in 1 mL of PBS and homogenized in a loose-fitting Teflon homogenizer. The homogenized sample was placed in quartz cuvettes and DHE fluorescence intensity determined using an excitation wavelength of 318 nm and emission wavelength of 380 nm.

Cholesterol Partitioning into HDL. NBD-cholesterol in dimethylformamide (DMF) solution was repeatedly added to 2 mL of PBS that contained 10 μg of HDL protein/mL of buffer. Fluorescence intensity (460 nm excitation and 530 nm emission) of the sample was recorded 3 min after the addition of the lipid. This time interval (3 min) was chosen because in a separate experiment we observed no increase in lipid fluorescence intensity between 2 and 5 min after addition to HDL (data not shown). Concentrations of NBD-cholesterol that were used were below its critical micelle concentration that we have previously reported (*25*). Ethanol (25 or 50 mM) was added to the sample 10 min before the addition of NBD-cholesterol.

Lipid Binding to ApoA-I and ApoA-I−PC Complexes. NBD-PC or NBD-cholesterol in a DMF solution was repeatedly added to 1 mL of PBS that contained 10 μg of apoA-I using procedures previously reported by our laboratory (*16*, *17*, *25*). The fluorescence intensity of the sample was recorded 5 min after addition of the lipid. This time interval (5 min) was chosen because in a separate experiment we observed no increase in lipid fluorescence intensity between 4 and 10 min after addition to apolipoprotein (data not shown). Concentrations of fluorescently labeled lipids used in the experiments were below their critical micelle concentrations (*25*). Ethanol (25 or 50 mM) was added to the samples 10 min before the addition of the fluorescently labeled lipids.

Tryptophan Fluorescence Polarization of ApoA-I and ApoE4. ApoA-I and apoE4 were dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 20 mM HEPES, with the pH adjusted to 7.4 with Tris base). ApoA-I or apoE4 (10 μg) was added to 1 mL of buffer and the extent of fluorescence polarization of tryptophan residues in the sample determined. Ethanol (25 and 50 mM) was added to the sample, and after 3 min, polarization was assessed. Polarization values were calculated as $(F_p - F_v)/(F_p + F_v)$, where F_p and F_v are tryptophan fluorescence intensities at parallel and vertical positions of polarizers, respectively.

Data Analysis. Student's *t* tests, Bonferonni-corrected for multiple comparisons, were used for statistical analyses.

RESULTS

Incorporation of Dehydroergosterol in CREF Cells. DHE has been previously used in several different studies of sterol dynamics (reviewed in refs *26*−*28*). DHE contains an endogenous fluorescent conjugated triene system in the B and C rings (*29*). DHE transport kinetics are analogous to those of cholesterol (*29*−*31*). The purpose of these baseline experiments was to determine the effects of DHE loading on CREF cell viability and the effects of DHE loading on cell membrane fluidity. Incorporation of DHE into cells was monitored by an increase in DHE fluorescence intensity. The fluorescence of nonincorporated DHE in the medium was negligible. When DHE is incorporated into cells, its fluorescence intensity greatly increases (Figure 1). Loading of cells with DHE was time-dependent with saturation approaching 24 h. We were able to obtain a reliable signal at a DHE concentration as low as 3 μg of DHE/mL of cell culture medium. After incubation for 24 h at a medium concentration of DHE of 3 μg/mL, fibroblasts contained 641.6 ± 35.1 ng of DHE/mg of fibroblast protein as measured by HPLC, and the DHE amount was less than 2% of the amount of total cell sterol. This amount of DHE did not affect cell viability, as shown by the absence of changes in LDH activity (absorbance at 490 nm was 0.1575 ± 0.0019 at 0 μg of DHE/mL of medium and 0.1601 ± 0.00174 at 3 μg of DHE/mL of medium). In addition, polarization of DPH in fibroblast did not differ between control (0.274 ± 0.006) and DHE-treated cells (0.270 ± 0.007). Sterol efflux experiments were performed using cells treated with 3 μg of DHE/mL of cell culture medium.

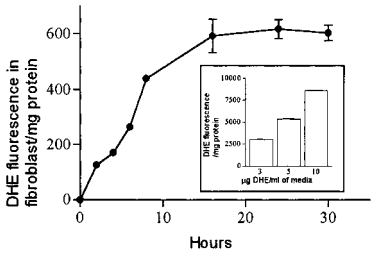

FIGURE 1: Incorporation of DHE into fibroblasts. In all experiments, confluent fibroblasts were used. DHE (3 μg/mL of cell culture medium) was added to cells, and at each time point, cells were processed and the DHE fluorescence was measured as described in Experimental Procedures. Data are means ± SEM ($n = 3$). (Inset) Confluent cells were incubated with different concentrations of DHE for 24 h. Cells were then harvested and processed, and DHE fluorescence was measured. Data are means ± SEM ($n = 4$).

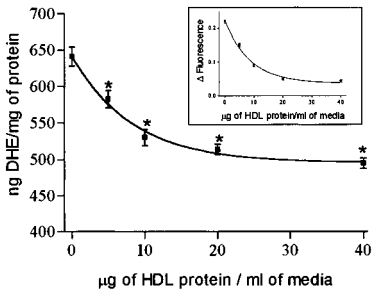

FIGURE 2: HDL-mediated efflux of DHE from fibroblasts. Confluent cells were incubated with 3 μg of DHE/mL of cell culture medium for 24 h. Cells were then washed twice using DHE-free medium and incubated with increasing concentrations of human plasma HDL for 2 h. Cells were then harvested and processed, and the sample was divided into two portions. One portion was used for DHE fluorescence measurements (inset), and the other portion was used to determine the amount of DHE by HPLC. Data are means ± SEM ($n = 3$).

Sterol Efflux to HDL and to the ApoA-I−PC Complex. Figure 2 shows the effects of increasing concentrations of HDL on sterol efflux from fibroblasts quantified by HPLC and DHE fluorescence intensity (Figure 2, inset). DHE was assessed in fibroblasts after HDL had been removed by centrifugation. HDL significantly removed DHE from fibroblasts, and this effect was maximal at 20 μg of HDL protein/mL of medium and did not change significantly when 40 μg of HDL protein/mL of medium was added (Figure 2). THe DHE fluorescence intensity paralleled the reduction

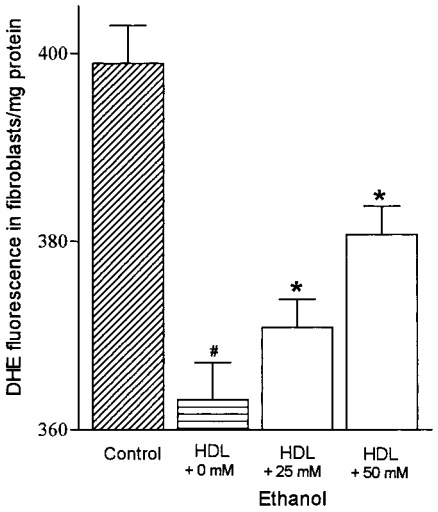

FIGURE 3: Effects of ethanol on HDL-mediated DHE efflux from fibroblasts. Confluent cells were incubated with 3 μg of DHE/mL of cell culture medium for 24 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25 or 50 mM ethanol or no ethanol, after which 30 μg of human plasma HDL protein/mL of medium was added and cells were incubated for 2 h. Cells were then harvested and processed, and DHE fluorescence was measured. The control cells (no HDL or ethanol) are cells that were incubated with DHE for 24 h where DHE fluorescence was measured as described. Data are means ± SEM ($n = 3$). # indicates $p \leq 0.001$ compared with control. * indicates $p \leq 0.01$ compared with the HDL without ethanol.

in the amount of DHE determined by HPLC in fibroblasts that were incubated with HDL (Figure 2). Ethanol in a dose-dependent manner (25 and 50 mM) significantly inhibited HDL-mediated cholesterol efflux as compared with HDL-mediated cholesterol efflux in the absence of ethanol (Figure 3). The fluorescence intensity of DHE in fibroblasts was not affected by ethanol when HDL was not present (fluorescence intensity conventional units, control, 356 ± 7.9; 50 mM ethanol, 359 ± 1.5), suggesting that ethanol was not directly interacting with DHE or affecting spontaneous desorption of sterol.

Figure 4 shows the effects of the apoA-I−PC complex (20 μg of protein/mL of cell culture medium) on sterol efflux from fibroblasts as measured by the decrease in fluorescence intensity of DHE remaining in the cells. There was a significant reduction in the amount of DHE in fibroblasts incubated with the apoA-I−PC complex when compared to cells not incubated with the apoA-I−PC complex (Figure 4). ApoA-I−PC-mediated sterol efflux was significantly inhibited by 50 mM ethanol (only concentration tested) compared with that in cells incubated with the apoA-I−PC complex in the absence of ethanol (Figure 4).

Cholesterol Partitioning into HDL. The fluorescence intensity of NBD-cholesterol added to HDL (10 μg of protein/mL) significantly increased when compared with that of NBD-cholesterol that was added to buffer alone (Figure 5). This increase in fluorescence intensity was interpreted as incorporation of NBD-cholesterol into HDL. Ethanol (50 mM) had no significant effect on incorporation of NBD-cholesterol into HDL (Figure 5). Fluorescence intensities of NBD-cholesterol and HDL compared with those of NBD-cholesterol and HDL incubated with 50 mM ethanol were similar at all concentrations of NBD-cholesterol that were tested (Figure 5).

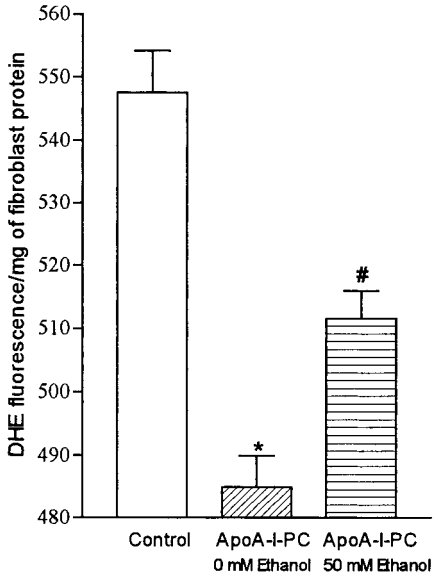

FIGURE 4: Effects of ethanol on apoA-I−PC-mediated DHE removal from fibroblasts. Cells were incubated with 3 μg of DHE/mL of cell culture medium for 24 h. Cells were then washed twice with DHE-free medium and incubated for 30 min with 50 mM ethanol or no ethanol. The apoA-I−PC complex (20 μg of protein/mL of medium) was then added to the cells and the mixture incubated for 2 h. Cells were then harvested and processed, and DHE fluorescence was measured. The control cells (no apoA-I−PC complex or ethanol) are cells that were incubated with DHE for 24 h where DHE was fluorescence measured as described. Data are means ± SEM ($n = 4$). * indicates $p \leq 0.01$ compared with control. # indicates $p \leq 0.01$ compared with the apoA-I−PC complex without ethanol.

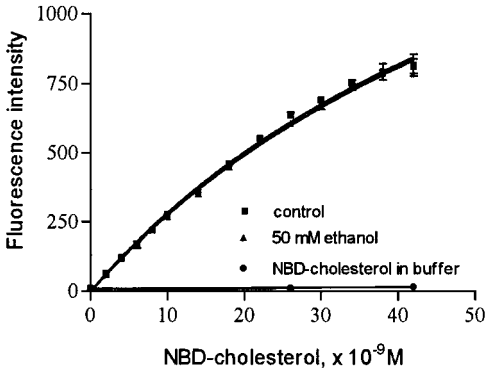

FIGURE 5: Incorporation of cholesterol into HDL and effects of ethanol. Human plasma HDL (10 μg of HDL protein/mL) was added to a buffer, and the sample was preincubated for 15 min with or without 50 mM ethanol. NBD-cholesterol (10^{-7} M solution in DMF) was then repeatedly added to the samples, and the fluorescence intensity of NBD was measured. The final concentration of DMF in the sample did not exceed 4.5 μM and had no effect on fluorescence: (■) control, (▲) 50 mM ethanol, and (●) NBD-cholesterol in buffer. Data are means ± SEM ($n = 5$).

Lipid Binding to ApoA-I and ApoA-I−PC Complexes. Lipid-free apoA-I has been reported to have a limited ability to bind nonpolar lipids (*32*). We found that binding of NBD-cholesterol to lipid-free apoA-I was not saturable and that ethanol (50 mM) had no effect on this process (data not shown). On the other hand, the fluorescence intensity of NBD-PC incubated with apoA-I was significantly increased compared with that of NBD-PC and buffer alone (Figure 6). Ethanol (25 and 50 mM) significantly decreased the fluorescence intensity of NBD-PC in the presence of apoA-I

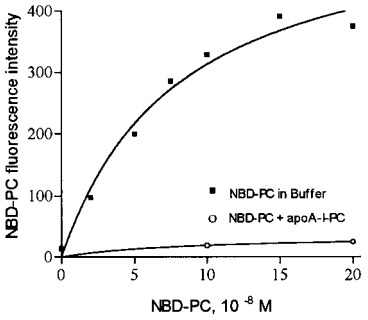

FIGURE 6: NBD-PC binding to apoA-I. ApoA-I (10 μg of protein/mL) in buffer was added to PBS at 36.5 °C. A NBD-PC solution in DMF (10^{-4} M) was then repeatedly added to the sample, and the fluorescence intensity was recorded. Data points on the curve are means ± SEM of five independent titrations.

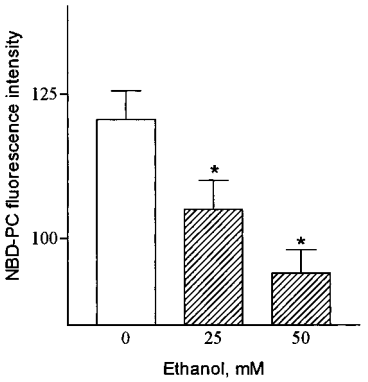

FIGURE 7: NBD-PC binding to apoA-I and effects of ethanol. ApoA-I was added to a buffer (10 μg of protein/mL of buffer), and the sample was preincubated for 15 min with or without ethanol. NBD-PC (2.5×10^{-7} M; 10^{-6} M solution in DMF) was then added to the control and ethanol (25 and 50 mM ethanol) samples, and the fluorescence intensity of NBD was measured. The final concentration of DMF in the sample did not exceed 2.5 μM and had no effect on fluorescence. Data are means ± SEM ($n = 4$). * indicates $p \leq 0.01$ compared with no ethanol.

as compared with that in the absence of ethanol (Figure 7). Ethanol at 25 and 50 mM had no effect on NBD-PC fluorescence in solution (fluorescence intensity, control, 21 ± 1.2; 25 mM ethanol, 23 ± 2.4; 50 mM ethanol, 20.4 ± 1.8), which would argue against a direct interaction of ethanol with NBD-PC.

PC complexed with apoA-I increases the level of cholesterol efflux as compared with lipid-free apoA-I (*12*). Data in Figure 8 show that the fluorescence intensity of NBD-cholesterol incubated with the apoA-I−PC complex increased with increasing amounts of sterol and that the increase in fluorescence intensity was saturable. Ethanol (25 and 50 mM) significantly reduced the fluorescence intensity of NBD-cholesterol incubated with the apoA-I−PC complex (Figure 9). Ethanol had no effect on NBD-cholesterol fluorescence in solution (fluorescence intensity, control, 12 ± 0.8; 25 mM ethanol, 11.9 ± 0.6; 50 mM ethanol, 12.1 ± 0.7).

Tryptophan Fluorescence Polarization of Apolipoproteins. One of the possible mechanisms of ethanol effects on HDL- and apoA-I−PC-mediated cholesterol efflux is direct interaction of ethanol with the apolipoprotein. ApoA-I is a major protein constituent of HDL. ApoA-I contains four tryptophan residues, and we utilized steady-state fluorescence polarization of tryptophan residues to establish ethanol interaction with apoA-I. Another apolipoprotein that is found in HDL

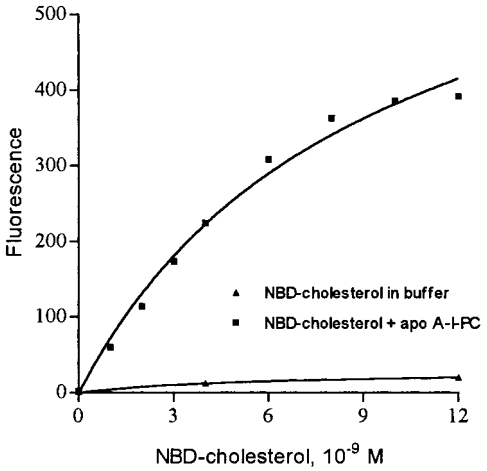

FIGURE 8: NBD-cholesterol incorporation into the apoA-I−PC complex. The apoA-I−PC complex (20 μg of protein/mL) was added to a buffer, and the sample was preincubated for 15 min. NBD-cholesterol (10^{-7} M solution in DMF) was then repeatedly added to the samples, and the fluorescence intensity of NBD was measured. The final concentration of DMF in the sample did not exceed 4.5 μM and had no effect on fluorescence: (▲) NBD-cholesterol in buffer and (■) NBD-cholesterol with the apoA-I−PC complex. Data are means ± SEM ($n = 4$).

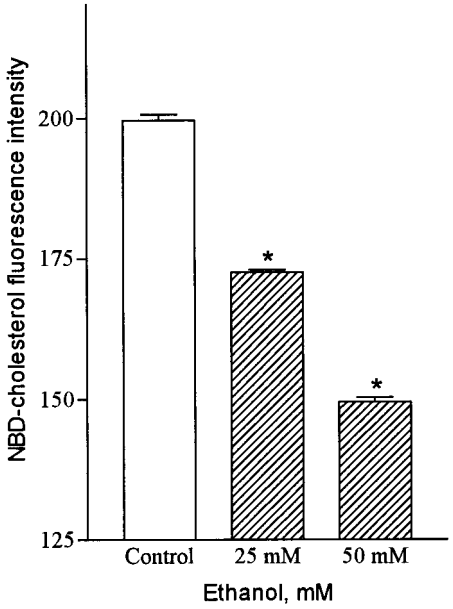

FIGURE 9: Effects of ethanol on NBD-cholesterol incorporation into the apoA-I−PC complex. The apoA-I−PC complex was added to a buffer (20 μg of complex protein/mL of buffer), and the sample was preincubated for 15 min with or without ethanol (25 and 50 mM). NBD-cholesterol (5×10^{-9} M; 10^{-7} M solution in DMF) was then added to the control and ethanol samples, and the fluorescence intensity of NBD was measured. The final concentration of DMF in the sample did not exceed 0.5 μM and had no effect on fluorescence. Data are means ± SEM ($n = 4$). * indicates $p \leq 0.01$ compared with the control condition.

is apoE. Although apoE-containing HDL represent a minor fraction of human HDL, its role in cholesterol transport is recognized. We measured effects of ethanol on tryptophan fluorescence polarization of one of the isoforms of apoE (apoE4). Data depicted in Figure 10 demonstrate a concentration-dependent effect of ethanol on apoA-I tryptophan fluorescence polarization. A significant increase in the level of polarization was observed as low as 25 mM ethanol (lowest concentration tested). A possible interpretation of

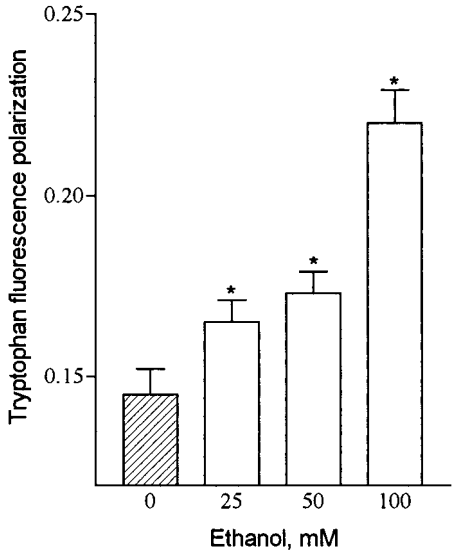

FIGURE 10: Steady-state fluorescence polarization of tryptophan residues in apoA-I and effects of ethanol. ApoA-I in buffer was added to PBS (10 μg of protein/mL) at 36.5 °C. Samples were then preincubated with or without ethanol (25, 50, or 100 mM) for 10 min, and the extent of tryptophan fluorescence polarization was measured. Data are means ± SEM ($n = 4$). * indicates $p \leq 0.01$ compared with no ethanol.

these data is that the ethanol-induced change in apoA-I tryptophan polarization resulted from ethanol altering the protein conformation. On the other hand, ethanol had no effect on tryptophan fluorescence polarization of apoE4 (control, level of polarization = 0.148 ± 0.011; 50 mM ethanol, level of polarization = 0.146 ± 0.007), suggesting a specific effect of ethanol on apoA-I. The tryptophan fluorescence intensity of apoA-I was not affected by ethanol, indicating that there was no direct interaction between ethanol and tryptophan residues.

DISCUSSION

This study tested the hypothesis that ethanol at concentrations observed in heavy drinkers modifies cholesterol efflux mediated by HDL and lipid-poor and -free apolipoproteins. In addition, direct effects of ethanol on apolipoprotein structure and lipid domains were examined as potential mechanisms that could explain effects of ethanol on cholesterol efflux. We found that ethanol at concentrations (25 and 50 mM) commonly observed in heavy drinkers significantly inhibited cholesterol efflux from fibroblasts to HDL and the apoA-I−PC complex. Ethanol had a differential effect on the interaction of cholesterol with HDL and the apoA-I−PC complex, leading us to the conclusion that the effects of ethanol on cholesterol efflux mediated by HDL involve interaction with the cell surface and that efflux mediated by the apoA-I−PC complex is a combination of aqueous diffusion and contact with the cell surface.

Two major mechanisms have been previously proposed to explain cholesterol efflux, an aqueous diffusion pathway and a pathway requiring interaction of HDL or lipid-poor or -free apolipoproteins with the membrane surface (*10*, *11*). Aqueous diffusion involves desorption of cholesterol from the cell plasma membrane, diffusion through the aqueous phase, and incorporation of cholesterol into an acceptor such as HDL. The second mechanism involves HDL or apolipoproteins that are lipid-free or -poor and requires direct interaction of the apolipoprotein with the plasma membrane. Ethanol significantly inhibited cholesterol efflux from fibroblasts to HDL and the apoA-I−PC complex.

If ethanol is affecting the aqueous diffusion pathway, ethanol could alter the incorporation of cholesterol, once it leaves the cell membrane, into HDL or the apoA-I−PC complex. Ethanol has been shown to inhibit binding of lipids, including cholesterol, to BSA and SCP-2 (*16*, *17*). Effects of ethanol on interaction of cholesterol with HDL or the apoA-I−PC complex were modeled by adding low concentrations of cholesterol to HDL and to the apoA-I−PC complex in the presence of ethanol. There were no significant differences between the amount of sterol that partitioned into HDL in the absence and in the presence of ethanol, but ethanol inhibited partitioning of cholesterol into the apoA-I−PC complex. Ethanol-induced inhibition of HDL-mediated cholesterol efflux may not involve the aqueous diffusion mechanism but may involve HDL interacting with the membrane. We did not examine the direct interaction of HDL with the membrane, but other studies provide support for such a mechanism. For example, efflux of cholesterol from cells to HDL has been shown to be associated with the expression of scavenger receptor class B type I (SR-BI) that is located in plasma membranes of some cells (*33*−*35*). Ethanol could interfere with binding of HDL to SR-BI by affecting the membrane lipid environment in which SR-BI is located or directly binding to the receptor. Effects of ethanol on membrane lipid structure are well-recognized (reviewed in refs *36* and *37*), and there are data showing that ethanol may directly interact with GABA, NMDA, and nicotinic acetylcholine receptors (*38*−*40*).

Ethanol on the other hand inhibited incorporation of cholesterol into the apoA-I−PC complex, suggesting that ethanol-induced inhibition of cholesterol efflux to the apoA-I−PC complex involves the aqueous diffusion pathway as compared with HDL. In addition, the mechanism of lipid-poor apolipoproteins directly interacting with the membrane cannot be ruled out with regard to the effects of ethanol on cholesterol efflux to the apoA-I−PC complex. Our findings that ethanol inhibited incorporation of cholesterol into the apoA-I−PC complex but not into HDL may have implications with respect to pre-β-HDL that have been previously described (*41*, *42*). ApoA-I−PC complexes have been reported to be similar in structural and functional properties to pre-β-HDL (*12*). Pre-β-HDL may be more susceptible to effects of ethanol in vivo than mature HDL. Additional potential mechanisms for the inhibitory effects of ethanol on cholesterol efflux are ethanol perturbation of the Golgi apparatus and interaction of ethanol with the ATP-binding cassette transporter (ABC1). Both the Golgi apparatus and ABC1 are involved in cholesterol efflux, and agents known to act on the Golgi apparatus and ABC1 inhibited cholesterol efflux (*43*, *44*). Lipid transport was recently reported to be disrupted in ABC1-deficient mice (*45*).

Incorporation of cholesterol into the apoA-I−PC complex was inhibited by ethanol. This effect of ethanol required the presence of PC. Ethanol had no effect on the interaction of NBD-cholesterol with lipid-free apoA-I, but ethanol significantly decreased the level of NBD-PC binding to apoA-I. It is becoming recognized that phospholipids may play an important role in apolipoprotein-mediated cholesterol efflux

(*10*−*12*). For example, it has been previously proposed that construction of apoA-I phospholipid complexes may be an initial and important step for apoA-I to effectively mediate cholesterol efflux (*12*). Ethanol would appear to interfere with this initial step in formation of apoA-I−PC complexes.

Modification of the apolipoprotein structure can alter lipid binding and lipid efflux (*11*, *14*, *18*). Ethanol-induced changes in function of HDL and the apoA-I−PC complex could be due in part to direct effects of ethanol on the apolipoprotein. Fluorescence analysis of tryptophan residues in proteins has been previously used to assess protein dynamics, including those of apolipoproteins (*19*, *46*−*48*). ApoA-I is a single polypeptide chain of 243 amino acids (*14*). There are four tryptophan residues located at positions 8, 50, 72, and 108. Ethanol (25, 50, and 100 mM) significantly increased the extent of apoA-I tryptophan fluorescence polarization. The apoA-I tryptophan fluorescence intensity was not affected by ethanol, indicating that ethanol did not quench apoA-I tryptophan fluorescence. It has recently been reported that the steady-state anisotropy of intrinsic tryptophan residues of lysozyme complexed with *N*-acetyl-D-glucosamine was increased and that the increase in anisotropy was due to the restricted motion of tryptophan (*19*). Ethanol may directly alter apoA-I structure, and this effect reduces the level of binding of lipids to the apolipoprotein. Tryptophan fluorescence polarization of HDL was not affected by ethanol. The apoA-I domain of HDL may be stabilized by making contact with lipids and resistant to effects of ethanol. Increasing the size of HDL particles reduced the magnitude of the effects of denaturation by guanidine hydrochloride (*48*). In the same study, it was shown that apoA-I was denatured almost immediately by guanidine hydrochloride. Ethanol had no effect on apoE4 tryptophan fluorescence polarization or intensity. The apoE4 tryptophan residues are located in a 22 kDa N-terminal domain that has been reported to be resistant to denaturation (*49*) and that we now show is resistant to ethanol perturbation.

In conclusion, we have shown that cholesterol efflux from fibroblasts to HDL and the apoA-I−PC complex was inhibited by ethanol concentrations that are routinely observed in plasma of heavy drinkers. While ethanol inhibited cholesterol efflux to HDL and the apoA-I−PC complex, the mechanisms of this inhibition would appear to be different. The inhibition of apoA-I−PC-mediated efflux involved the aqueous diffusion mechanism. Inhibition of HDL-mediated efflux did not appear to involve aqueous diffusion. ApoA-I structure as revealed by steady-state polarization of tryptophan residues was more affected by ethanol than by HDL. ApoA-I−PC complexes have been reported to be similar in structural and functional properties to pre-β-HDL (*12*). Pre-β-HDL may be more susceptible to effects of ethanol in vivo than mature HDL. The study presented here shows that ethanol directly inhibited cholesterol efflux and modified apolipoprotein structure. Recently, it was reported that HDL of alcoholic patients were less efficient in removing cholesterol from mouse macrophages and in the uptake of cholesterol by HepG2 cells than HDL from control subjects (*50*). In that study, cells were not incubated with ethanol. Therefore, reverse cholesterol transport in heavy drinkers and alcoholics may be inhibited by acute ethanol consumption and modification of HDL associated with chronic ethanol consumption.

REFERENCES

1. Eagles, C. J., and Martin, U. (1998) *J. Clin. Pharm. Ther. 23*, 1−9.
2. Klatsky, A. L. (1999) *Alcohol Res. Health 23*, 15−23.
3. Sesso, H. D., and Gaziano, J. M. (1999) *Curr. Opin. Nephrol. Hypertens. 8*, 353−357.
4. Castelli, W. P., Doyle, J. T., Gordon, T., Hames, C. G., Hjortland, M. C., Hulley, S. B., Kagan, A., and Zukel, W. J. (1977) *Lancet 2*, 153−155.
5. Klatsky, A. L. (1994) *Alcohol.: Clin. Exp. Res. 18*, 88−96.
6. Frohlich, J. J. (1996) *Clin. Chim. Acta 246*, 39−49.
7. Srivastava, L. M., Vasisht, S., Agarwal, D. P., and Goedde, H. W. (1994) *Alcohol Alcohol. (Oxford) 29*, 11−24.
8. Goldberg, D. M., Hahn, S. E., and Parkes, J. G. (1995) *Clin. Chim. Acta 237*, 155−187.
9. Hojnacki, J. L. (1994) *Nutr. Res. 14*, 1241−1295.
10. Rothblat, G. H., de la Llera-Moya, M., Atger, V., Kellner-Weibel, G., Williams, D. L., and Phillips, M. C. (1999) *J. Lipid Res. 40*, 781−796.
11. Mendez, A. J. (1997) *J. Lipid Res. 38*, 1807−1821.
12. Zhao, Y., Sparks, D. L., and Marcel, Y. L. (1996) *J. Biol. Chem. 271*, 25145−25151.
13. Fielding, C. J., and Fielding, P. E. (1995) *J. Lipid Res. 36*, 211−228.
14. Brouillette, C. G., and Anantharamaiah, G. M. (1995) *Biochim. Biophys. Acta 1256*, 103−129.
15. Daniels, C. K., and Goldstein, D. B. (1982) *Mol. Pharmacol. 21*, 694−700.
16. Avdulov, N. A., Chochina, S. V., Daragan, V. A., Schroeder, F., Mayo, K. H., and Wood, W. G. (1996) *Biochemistry 35*, 340−347.
17. Avdulov, N. A., Chochina, S. V., Igbavboa, U., Warden, C., Schroeder, F., and Wood, W. G. (1999) *Biochim. Biophys. Acta 1437*, 37−45.
18. Burgess, J. W., Frank, P. G., Franklin, V., Liang, P., McManus, D. C., Desforges, M., Rassart, E., and Marcel, Y. L. (1999) *Biochemistry 38*, 14524−14533.
19. Nishimoto, E., Yamashita, S., Szabo, A. G., and Imoto, T. (1998) *Biochemistry 37*, 5599−5607.
20. Igbavboa, U., Avdulov, N. A., Schroeder, F., and Wood, W. G. (1996) *J. Neurochem. 66*, 1717−1725.
21. Igbavboa, U., Avdulov, N. A., Chochina, S. V., and Wood, W. G. (1997) *J. Neurochem. 69*, 1661−1667.
22. Schroeder, F., Butko, P., Nemecz, G., and Scallen, T. J. (1990) *J. Biol. Chem. 265*, 151−157.
23. Avdulov, N. A., Wood, W. G., and Harris, R. A. (1994) *Alcohol.: Clin. Exp. Res. 18*, 53−59.
24. Avdulov, N. A., Chochina, S. V., Igbavboa, U., O'Hare, E. O., Schroeder, F., Cleary, J. P., and Wood, W. G. (1997) *J. Neurochem. 68*, 2086−2091.
25. Avdulov, N. A., Chochina, S. V., Igbavboa, U., Vassiliev, A. V., and Wood, W. G. (1997) *J. Neurochem. 69*, 1746−1752.
26. Schroeder, F. (1985) *Subcell. Biochem. 11*, 51−100.
27. Schroeder, F., Jefferson, J. R., Kier, A. B., Knittel, J., Scallen, T. J., Wood, W. G., and Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196*, 235−252.
28. Schroeder, F., Frolov, A. A., Murphy, E. J., Atshaves, B. P., Pu, L., Wood, W. G., Foxworth, W. B., and Kier, A. B. (1996) *Proc. Soc. Exp. Biol. Med. 213*, 150−177.
29. Kier, A. B., Sweet, W. D., Cowlen, M. S., and Schroeder, F. (1986) *Biochim. Biophys. Acta 861*, 287−301.
30. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) *Biophys. J. 75*, 1915−1925.
31. Yeagle, P. L. (1982) *Biochemistry 21*, 1249−1254.
32. Titov, V. N. (1997) *Biochemistry (Moscow) 62*, 3−19.
33. Acton, S. A., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) *Science 271*, 518−520.
34. Rigotti, A., Edelman, E. R., Seifert, P., Iqbal, S. N., DeMattos, R. B., Temel, R. E., Krieger, M., and Williams, D. L. (1996) *J. Biol. Chem. 271*, 33545−33549.
35. Jian, B., de la Llera-Moya, M., Ji, Y., Wang, N., Phillips, M. C., Swaney, J. B., Tall, A. R., and Rothblat, G. H. (1998) *J. Biol. Chem. 273*, 5599−5606.

36. Wood, W. G., Rao, A. M., Schroeder, F., and Igbavboa, U. (1993) in *Alcohol, Cell Membranes, and Signal Transduction in Brain* (Alling, C., Diamond, I., Leslie, S. W., Sun, G. Y., and Wood, W. G., Eds.) pp 13−32, Plenum Press, New York.
37. Wood, W. G., Schroeder, F., Rao, A. M., Igbavboa, U., and Avdulov, N. A. (1996) in *Pharmacological Effects of Ethanol on the Nervous System* (Deitrich, R. A., and Erwin, V. G., Eds.) pp 13−27, CRC Press, Boca Raton, FL.
38. Mihic, S. J., Ye, Q., Wick, M. J., Koltchine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., Harris, R. A., and Harrison, N. L. (1997) *Nature 389*, 385−389.
39. Li, C., Peoples, R. W., and Weight, F. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8200−8204.
40. Wood, S. C., Forman, S. A., and Miller, K. W. (1991) *Mol. Pharmacol. 39*, 332−338.
41. Kunitake, S. T., La Sala, K. J., and Kane, J. P. (1985) *J. Lipid Res. 26*, 549−555.
42. Castro, G. R., and Fielding, C. J. (1988) *Biochemistry 27*, 25−29.
43. Mendez, A. J. (1995) *J. Biol. Chem. 270*, 5891−5900.
44. Lawn, R. M., Wade, D. P., Garvin, M. R., Wang, X., Schwartz, K., Porter, J. G., Seilhamer, J. J., Vaughan, A. M., and Oram, J. F. (1999) *J. Clin. Invest. 104*, 2−3.
45. Orso, E., Broccardo, C., Kaminski, W. E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M. F., Rothe, G., Lackner, K. J., Chimini, G., and Schmitz, G. (2000) *Nat. Genet. 24*, 192−196.
46. Kemple, M. D., Yuan, P., Nollet, K. E., Fuchs, J. A., Silva, N., and Prendergast, F. G. (1994) *Biophys. J. 66*, 2111−2126.
47. Jonas, A., Kézdy, K. E., and Wald, J. W. (1989) *J. Biol. Chem. 264*, 4818−4824.
48. Jonas, A., Wald, J. W., Harms Toohill, K. L., Krul, E. S., and Kézdy, K. E. (1990) *J. Biol. Chem. 265*, 22123−22129.
49. Wetterau, J. R., Aggerbeck, L. P., Rall, S. C., and Weisgraber, K. H. (1988) *J. Biol. Chem. 263*, 6240−6248.
50. Rao, M. N., Liu, Q.-H., Seef, L. B., Strader, D. B., Marmillot, P. R., and Lakshman, M. R. High-density lipoproteins from human alcoholics exhibit impaired reverse cholesterol transport function, *Metabolism* (in press).

BI0008534