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13. ABSTRACT *(Maximum 200 Words)*
The focus of this grant is on mechanisms of alcohol induced disruption of cellular cholesterol transport and distribution. Alcohol at concentrations observed in problem drinkers and alcoholics (for example an individual consuming either 6 beers, or 6 one oz shots of whiskey, or 6 glasses of wine) has a profound and multifaceted effect on cellular regulation of cholesterol. Ethanol significantly inhibited cholesterol efflux from human aortic smooth muscle cells to HDL. The Golgi complex that is important in regulation of cholesterol efflux is a target of ethanol as revealed by confocal microscopy. Preliminary data indicate that acute and chronic effects of ethanol on cholesterol efflux from human aortic smooth muscle cells to HDL have opposite effects. LDL-mediated cholesterol uptake is stimulated by ethanol. Alcohol results in more cholesterol being taken into cells and less cholesterol being removed from cells. These findings have several important implications. Cholesterol content in cells of problem drinkers and alcoholics may be elevated as compared to control subjects and such a difference would not be detected by routine clinical assays for cholesterol. Cholesterol is essential for the normal function of cells. Alcohol-induced changes in cell cholesterol content could have profound pathophysiological consequences on cell function.

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INTRODUCTION

Molecular mechanisms that are involved in effects of moderate and heavy alcohol consumption on cholesterol transport and cellular cholesterol distribution are not well-understood. We propose that ethanol modifies high density lipoprotein (HDL)-mediated cholesterol efflux from cells and low density lipoprotein (LDL)-mediated cholesterol influx into cells. These actions of ethanol alter the intracellular distribution of cholesterol leading to cellular dysfunction. Potential mechanisms that may explain effects of ethanol on cholesterol transport and distribution include ethanol perturbation of lipoprotein structure, LDL receptor function and expression, perturbation of the Golgi complex and signaling pathways involved in reverse cholesterol transport. These potential mechanisms are being studied in this grant that consist of three major objectives: **Objective 1.** to determine mechanisms of ethanol-induced increase in LDL receptor-mediated cholesterol influx; **Objective 2.** to determine mechanisms of ethanol-induced alterations in the intracellular distribution of cholesterol; and **Objective 3.** to determine mechanisms of ethanol-induced perturbation of HDL-mediated cholesterol efflux from cells. Studies are being conducted using a combination of fluorescence spectroscopy, confocal microscopy and cell culture techniques. Human aortic smooth muscle cells, human endothelial cells, and human fibroblasts are being used in the experiments.

BODY OF REPORT

In the review of our application, it was recommended that we include human aortic smooth muscle cells because those cells are the site of cholesterol accumulation in the atherosclerotic plaque. A revised Statement of Work to reflect addition of the cells was requested and this was submitted. This recommendation was very astute and human aortic smooth muscle cells are part of the project in addition to human coronary artery endothelial cells and human fibroblasts.

Dehydroergosterol, HDL, and Human Aortic Smooth Muscle Cells. We have successfully established the use of human aortic smooth muscle cells (HASMC) in our laboratory. As a first step, basic experiments on optimal conditions of dehydroergosterol (DHE) incorporation and HDL-mediated efflux were completed. DHE is a sterol occurring in yeast and it is very similar to cholesterol in structure and function [1;2]. This sterol is naturally fluorescent (it contains an endogenous fluorescent conjugated triene system in the B and C rings) and has been used in several studies of cholesterol dynamics. We had previously reported that DHE at a concentration of 3μg/ml of media was efficiently taken up by fibroblasts and that sterol transport could be quantified by an increase in fluorescence intensity of DHE [3]. Cholesterol transport dynamics are dependent on cell type. Three different concentrations of DHE were incubated with HASMC for 18 h. The fluorescence intensity of DHE was measured in cells and the data are shown in Figure 1. It can be seen that of three different concentrations of DHE (1,2,3 μg/ml media), 3ug/ml produced a robust increase in DHE fluorescence intensity. All experiments will be performed with a DHE concentration of 3ug/ml of media. The next experiments determined the time point following incubation of cells with DHE that incorporation plateaued. Cells were incubated with DHE for 24 h and at various times incorporation of DHE into cells was measured by fluorescence intensity of DHE. Figure 2 shows that loading of cells with DHE was time-dependent with saturation approaching 18-24 h. Subsequent experiments used an incubation time of 18 h for DHE incorporation into cells. Experiments were then performed to determine the

optimal concentration of HDL required for sterol efflux. Different concentrations of HDL (10,20,30,40 μg/ml media) were incubated with HASMC that had been loaded with DHE. Figure 3 shows that significant cholesterol efflux occurred at HDL concentrations between 20-40 μg/ml of buffer. A HDL concentration of 30 μg was used in all subsequent experiments.

Ethanol Inhibits HDL-Mediated Cholesterol Efflux from HASMC. Cells that had been loaded with DHE were incubated with ethanol (25, 50, 75 mM) for 30 min. This procedure allowed ethanol to equilibrate and has been used previously by our laboratory [3]. After 30 min, HDL was added to cells for a 2 h period at which time cells were pelleted and fluorescence of DHE remaining in cells determined. Ethanol significantly inhibited HDL-mediated cholesterol efflux (Figure 4). Inhibition was seen at 50 and 75 mM. We have previously reported that 25 mM ethanol [3] inhibited cholesterol efflux in human fibroblasts but this effect was not observed with HASMC.

Ethanol, Cholesterol, and the Golgi Complex. We showed that HDL-mediated cholesterol efflux was inhibited by ethanol (Figure 4). Cholesterol efflux is controlled by several mechanisms and the Golgi complex is important in regulation of cholesterol efflux [4]. There is evidence that agents known to disrupt Golgi function can inhibit cholesterol efflux [5]. We have hypothesized that ethanol could perturb Golgi structure or specific signaling pathways that regulate cholesterol efflux. To begin to understand the behavior of ethanol on the Golgi complex, we determined effects of ethanol on cholesterol content of the Golgi complex in fibroblasts using confocal microscopy and densitometric analysis. The fluorescent cholesterol analogue, NBD-cholesterol, and a fluorescent label for the Golgi complex, BODIPY TR ceramide were used. In some experiments zinc was used that has been previously shown to stimulate cholesterol transport. Figure 5 shows localization of cholesterol in Golgi of fibroblasts. Panels of Figure 5 show colocalization of the two fluorescent probes in control cells (panel C) cells incubated with 40 mM ethanol (panel F) and 80 mM ethanol (panel I). The reduction in fluorescence intensity in panels F and I are indicative of a reduction in Golgi cholesterol content. Densitometric analysis revealed that both 40 mM and 80 mM ethanol significantly reduced cholesterol content in the Golgi complex as compared to control cells (Table 1). Figure 6 contains confocal images of effects of zinc on cholesterol in the Golgi complex and the interaction of zinc and ethanol. Zinc significantly increased cholesterol in the Golgi complex and had a small but significant effect on inhibiting effects of ethanol (Table 1). These data clearly demonstrate that cholesterol in the Golgi complex is a target of ethanol.

The Golgi complex is involved in transport of lipids and proteins to the plasma membrane. There is evidence that disruption of Golgi structure will interfere with cholesterol efflux [4;6]. The exact mechanism that explains how disruption of the Golgi complex affect cholesterol efflux is not well-understood. A potential mechanism may involve the actions of two phospholipases, phosphatidylcholine-phospholipase C (PC-PLC) and phosphatidylcholine-phospholipase D (PC-PLD) [7]. HDL and apoA-1 are thought to stimulate a G-protein coupled receptor that induces hydrolysis of PC by PC-PLC and PLD. Stimulation of these pathways increases cholesterol efflux. It has been proposed that phosphatidic acid that is a metabolite of PC hydrolysis by PC-PLD may be involved in regulation of cholesterol efflux. Alcohols including ethanol block the formation of PA by PLD and instead produce phosphatidylethanol (Petoh) by a phosphatidyl-transfer reaction. Preliminary data revealed that propranolol stimulated cholesterol efflux to HDL

as compared with HDL alone. It is predicted that propranolol may inhibit effects of ethanol on cholesterol efflux. Ethanol may also directly act on PC-PLC and PC-PLD and alter the structure of the two phospholipases. We propose that the mechanism of effects of ethanol on cholesterol efflux is multifaceted and involves at least five major mechanisms as shown in Figure 7. Ethanol directly perturbs apolipoprotein structure and plasma membrane structure and receptors (1 & 2); ethanol may alter PC-PLC and PC-PLD activity (3); reduced production of PA and production of Petoh (4) that in turn reduces release of cholesterol from the Golgi complex by perhaps acting on lipid rafts (5).

Ethanol Stimulates LDL-Mediated Cholesterol Influx and Alters Apolipoprotein B Structure. Ethanol also acted on LDL-mediated cholesterol uptake. Ethanol at concentrations (25 & 50 mM) observed in heavy drinkers significantly ($p \leq 0.005$) enhanced the uptake of low density lipoproteins (LDL) containing fluorescent labeled sterol into cells (Figure 8). This action of ethanol on LDL involved ethanol directly acting on the major apolipoprotein of LDL, apolipoprotein B (apoB). Ethanol decreased the fluorescence polarization of tryptophan residues of apoB revealing that ethanol was modifying protein conformation (Figure 9). Ethanol-induced reduction in polarization indicates a less restrictive motion of apoB tryptophan residues. Effects of ethanol on apoB structure may provide a more favorable environment for LDL to bind to the LDL receptor and increase cholesterol influx.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of human aortic smooth muscle cells in studies of cholesterol transport as recommended by the reviewers.
- Ethanol significantly inhibited cholesterol efflux from human aortic smooth muscle cells to HDL.
- The Golgi complex that is important in regulation of cholesterol efflux is a target of ethanol as revealed by confocal microscopy.
- PC-PLC and PC-PLD may be involved in actions of ethanol on cholesterol efflux.
- Mechanisms of ethanol effects on cholesterol efflux of cells involve several in contrast to a single mechanism.

REPORTABLE OUTCOMES

Wood, W.G., Avdulov, N.A., Chochina, S.V., and Igbavboa, U. Lipid carrier proteins and ethanol. *Journal of Biomedical Science*, 2001, 8, 114-118.

Igbavboa, U., Kiss, Z., and Wood, W. G. Cholesterol in Golgi complex is modified by ethanol as revealed by confocal microscopy. *FASEB Journal*, 2001, *15*, A529.

Igbavboa, U. and Wood, W.G. Ethanol perturbation of cholesterol efflux from human aortic smooth muscle cells to HDL: Role of the Golgi complex. *Biochemistry*, in preparation.

CONCLUSIONS

Cholesterol is important in regulation of cell structure and function. Equally important is the role that cholesterol plays in vascular disease. The focus of this grant is on mechanisms of alcohol induced disruption of cellular cholesterol transport and distribution. Our findings to date demonstrate that alcohol at concentrations observed in problem drinkers and alcoholics (for example an individual consuming either 6 beers, or 6 one oz shots of whiskey, or 6 glasses of wine) has a profound and multifaceted effect on cellular regulation of cholesterol. Alcohol results in more cholesterol being taken into cells and less cholesterol being removed from cells. These findings have several important implications. The clinical determination of cholesterol is based on amount of cholesterol in the serum (total cholesterol, LDL-cholesterol, HDL-cholesterol) but not the amount of cholesterol in cells. Cholesterol content in cells of problem drinkers and alcoholics may be elevated as compared to control subjects and such a difference would not be detected by routine clinical assays for cholesterol. Cholesterol is essential for the normal function of cells. Alcohol-induced changes in cell cholesterol content could have profound pathophysiological consequences on cell function.

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Appendices

Figures 1-9

Table 1

Reprints

Figure Legends

Figure 1. Incorporation of Different Amounts of DHE into Human Aortic Smooth Muscle Cells (HASMC). Cells were incubated with different amounts of DHE for 18 h. Cells were then pelleted and fluorescence intensity of DHE in the cells was determined. Data are the means ± SEM of DHE in cells (3- 4 experiments.

Figure 2. Effects of Incubation Time on DHE Incorporation into HASMC. Cells were incubated with DHE (3μg/ml of media) for different time periods after which cells were pelleted and fluorescence intensity of DHE determined. Data are the means ± SEM of DHE in cells (3- 4 experiments.

Figure 3. HDL-Mediated DHE Efflux from HASMC. Cells were incubated with DHE (3μg/ml of media) for 18 h. Different amounts of HDL were then added for 2 h. Cells were then pelleted and fluorescence intensity of DHE in the cells was determined. Data are the means ± SEM of DHE in cells (3- 4 experiments). $^{*}p \leq 0.02$ as compared to cells not incubated with HDL.

Figure 4. Ethanol Inhibits HDL-Mediated Efflux from HASMC. Cells were incubated with DHE (3μg/ml of media) for 18 h at which time the cells were rinsed and different ethanol concentrations of ethanol were added. After 30 min, HDL (30μg/ml of media) were incubated with the cells for 2 h. Cells were then pelleted and DHE fluorescence intensity determined in cells. Data are the means ± SEM of DHE in cells (3- 4 experiments). $^{*}p \leq 0.0001$ as compared to the control condition (no HDL or ethanol). $^{+}p \leq 0.04$ as compared HDL without ethanol.

Figure 5. Effects of Ethanol on Localization of Cholesterol in Golgi of Fibroblasts using NBD-Cholesterol and BODIPY CR Ceramide. Cells were incubated with NBD-cholesterol for 1 h and then incubated with ethanol (40 or 80 mM) for 2 h and then incubated with BODIPY CR ceramide for 1 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde and mounted to be used for quantification of cholesterol using confocal microscopy and densitometric analysis. Control (panels A,B,C); 40 mM ethanol (panels D,E,F); 80 mM ethanol (panels G,H,I).

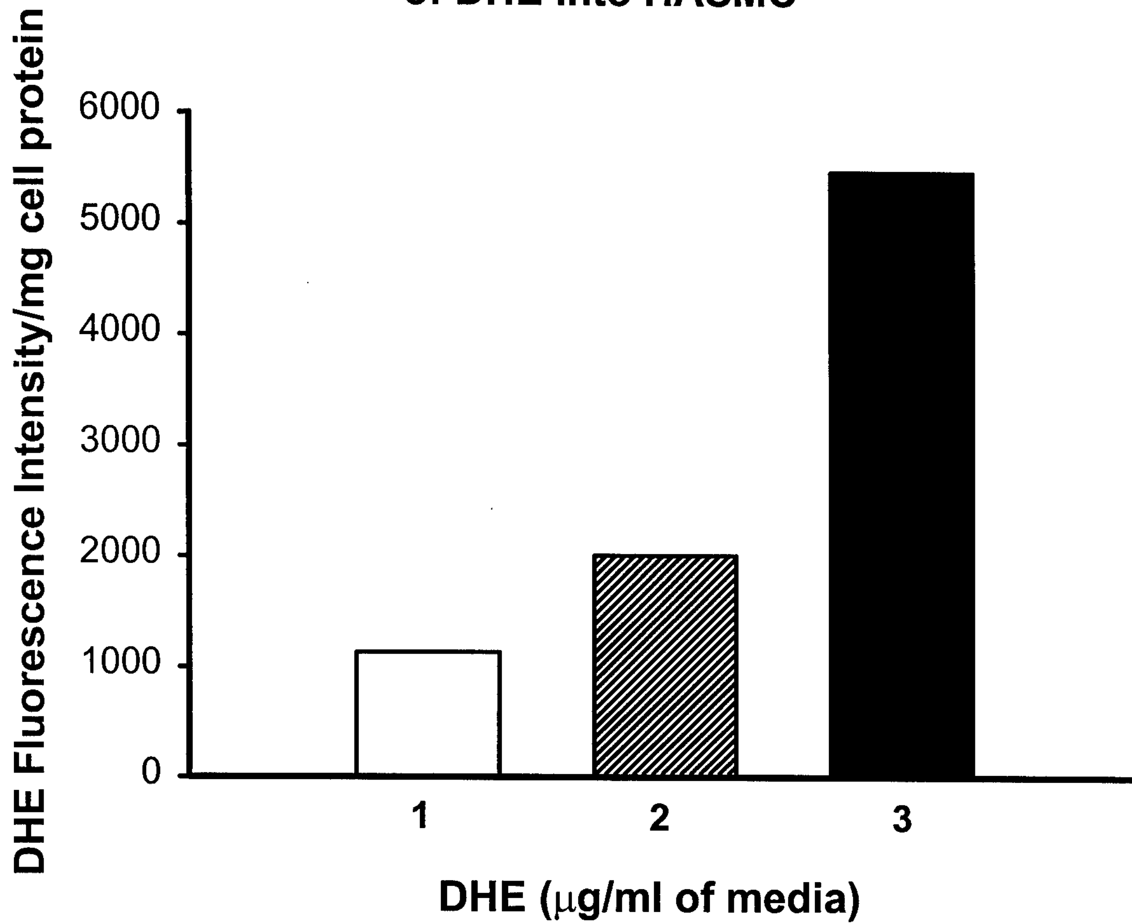
Figure 6. Effects of Ethanol and Zinc on Localization of Cholesterol in Golgi of Fibroblasts using NBD-Cholesterol and BODIPY CR Ceramide. Procedures were the same as described in the legend for Figure 5 with the exception that zinc (Zn^{2+}) was added to cells in some experiments. Control (panels A,B,C); Zn^{2+} (panels D,E,F); Zn^{2+} & 40 mM ethanol (panels G,H,I); Zn^{2+} & 80 mM ethanol (panels J,K,L).

Figure 7. Potential Mechanisms of Ethanol Effects on Cholesterol Efflux from Cells. . Ethanol directly perturbs apolipoprotein structure and plasma membrane structure and receptors(1 & 2); ethanol may alter PC-PLC and PC-PLD activity (3); reduced production of PA and production of Petoh (4) that in turn reduces release of cholesterol from the Golgi complex by perhaps acting on lipid rafts (5).

Figure 8. Effects of Ethanol on LDL-Mediated DHE Influx into Fibroblasts. DHE was incorporated into human plasma LDL. Cells were then incubated with LDL in the presence or absence of 25 and 50 mM ethanol for 5 h after which time cells were pelleted and fluorescence intensity of DHE measured. Data are the means ± SEM of DHE in cells (n = 4 experiments). $^{*}p \leq 0.005$ as compared with LDL control.

Figure 9. Ethanol and Tryptophan Fluorescence Polarization of apoB. Steady-state polarization was measured using tryptophan residues of apoB excited at 286 nm and emission at 335 nm in the presence and absence of 50 and 100 mM ethanol at 37°C. Data are means ± SEM of polarization values for apoB tryptophan residues (n = 5 experiments). $^{*}p \leq 0.001$ as compared with 0 ethanol.

Figure 1. Incorporation of Different Amounts of DHE into HASMC



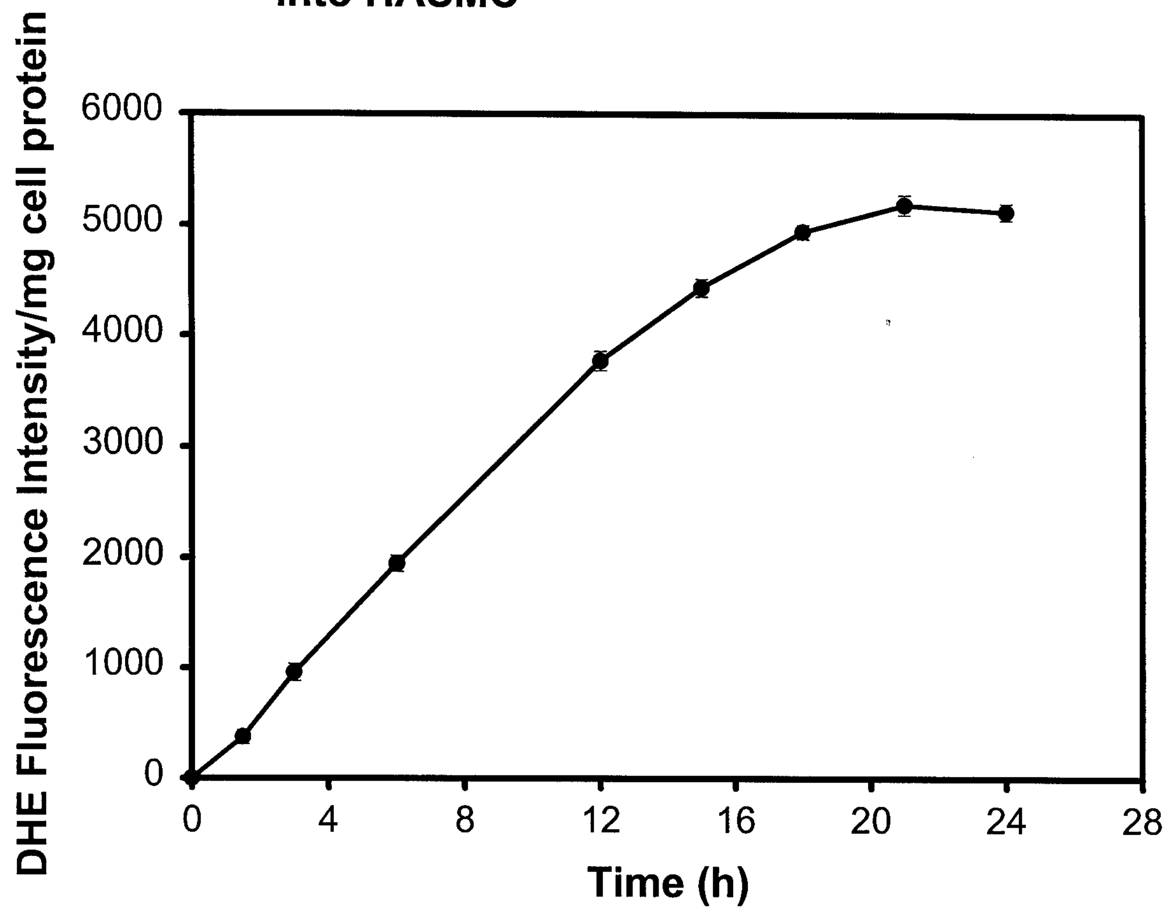


Figure 2. Effects of Incubation Time on DHE Incorporation into HASMC

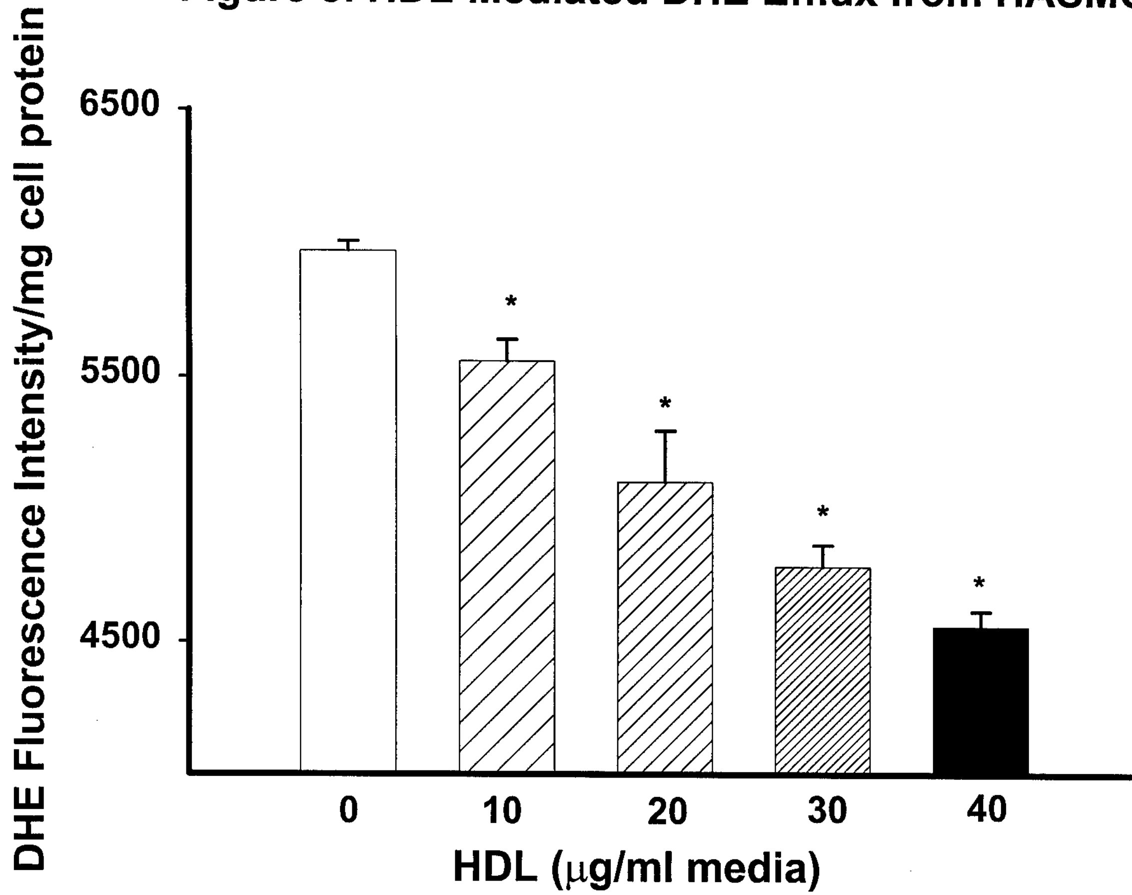
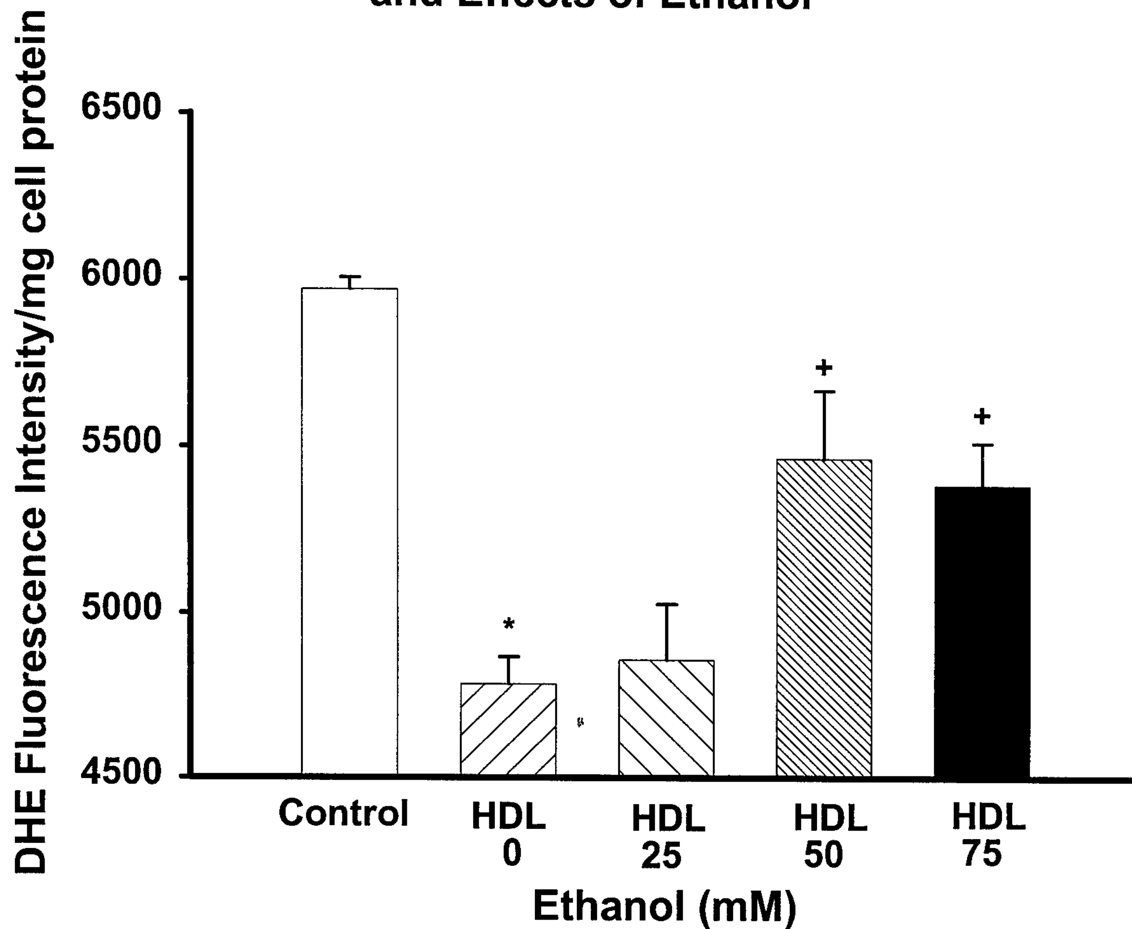


Figure 3. HDL-Mediated DHE Efflux from HASMC

*$p \leq 0.02$ as compared to cells not incubated with HDL

Figure 4. HDL-Mediated DHE Efflux from HASMC and Effects of Ethanol



*$p < 0.0001$ as compared to control.
+$p < 0.04$ as compared to HDL and 0 ethanol.

Control

40 mM EtOH added

80 mM EtOH added

A B C

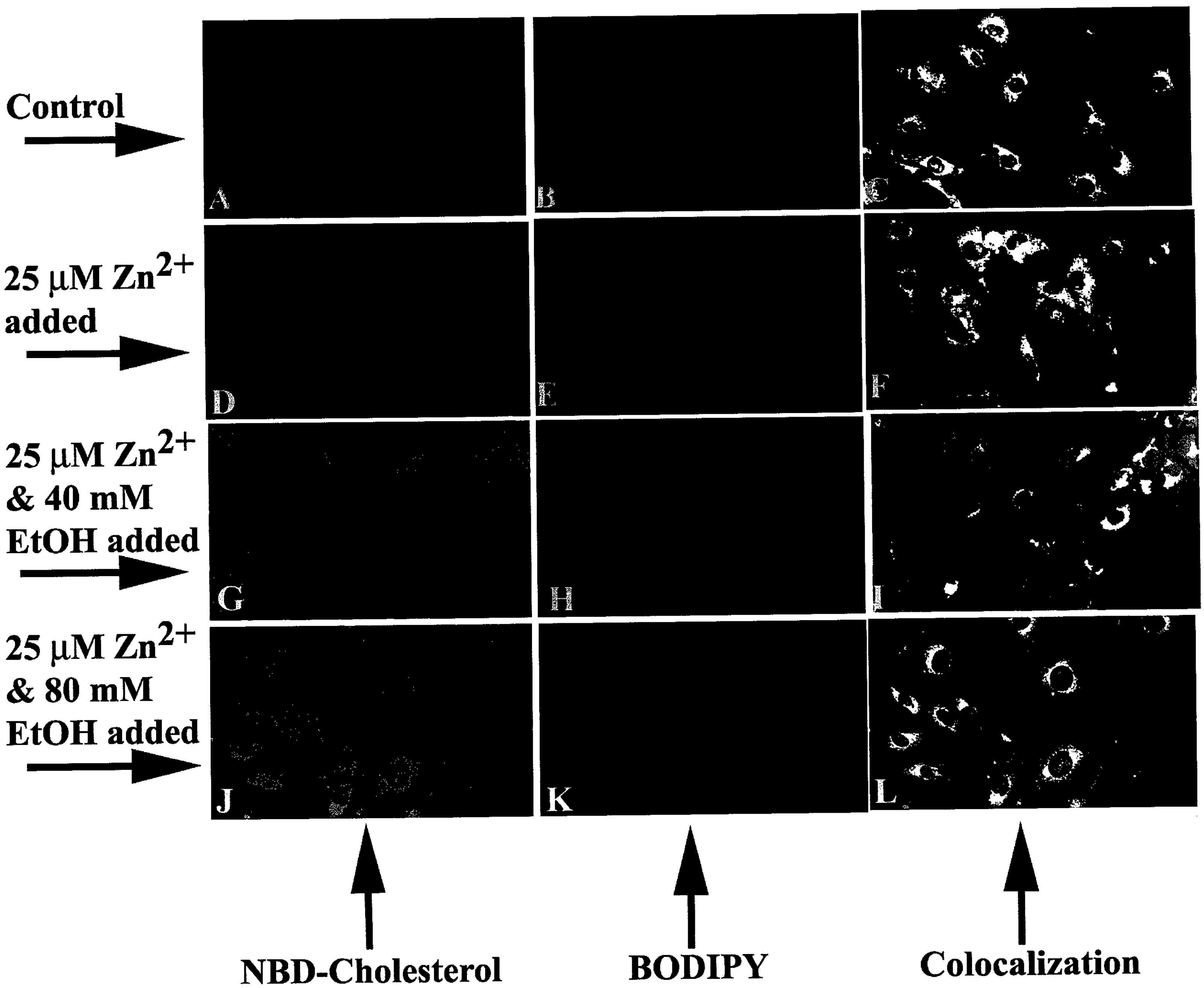
D E F

G H I

NBD-Cholesterol

BODIPY

Colocalization


Control
25 μM Zn2+ added
25 μM Zn2+ & 40 mM EtOH added
25 μM Zn2+ & 80 mM EtOH added
A
B
C
D
E
F
G
H
I
J
K
L
NBD-Cholesterol
BODIPY
Colocalization

Ethanol and Cholesterol Efflux

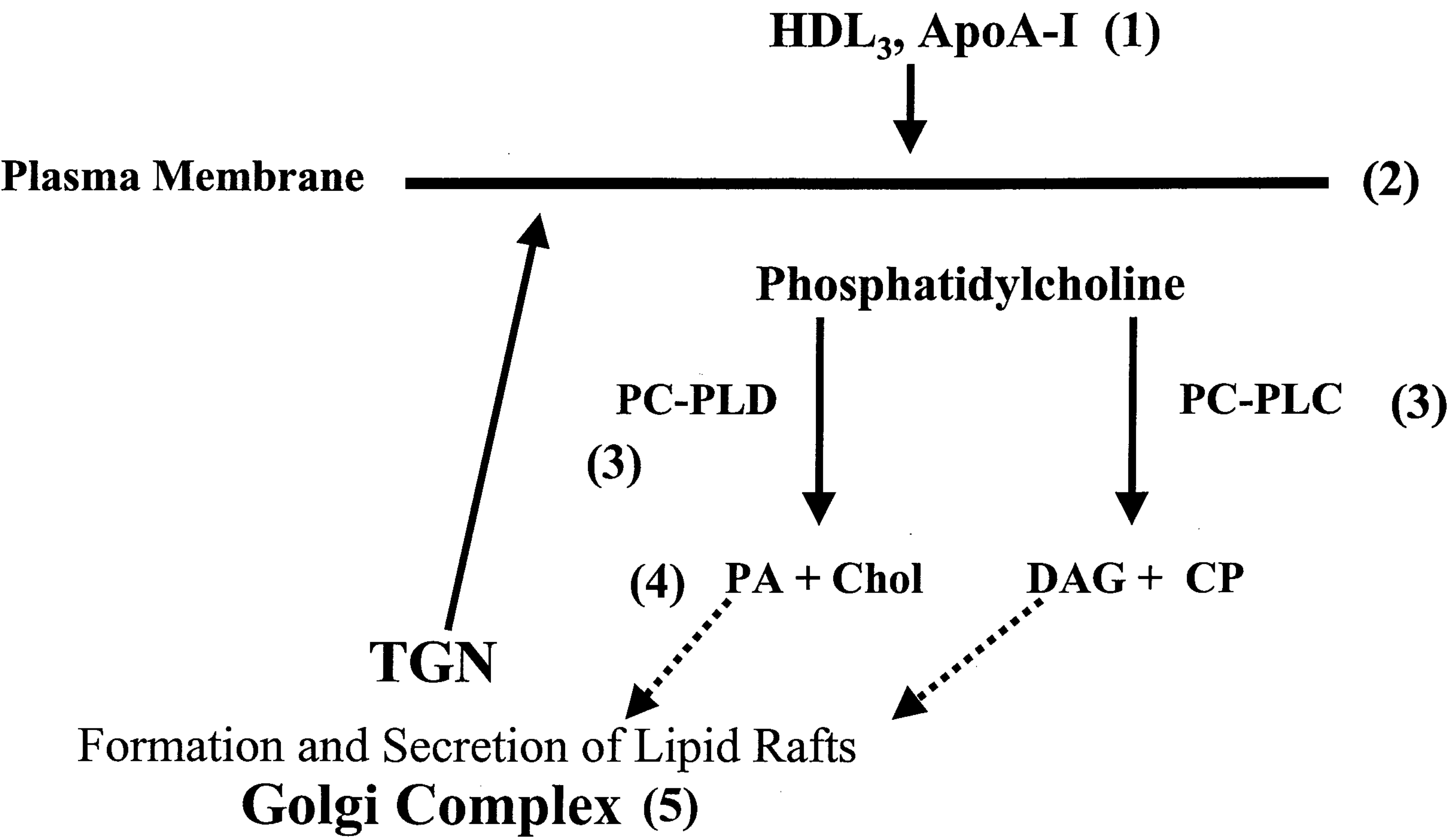
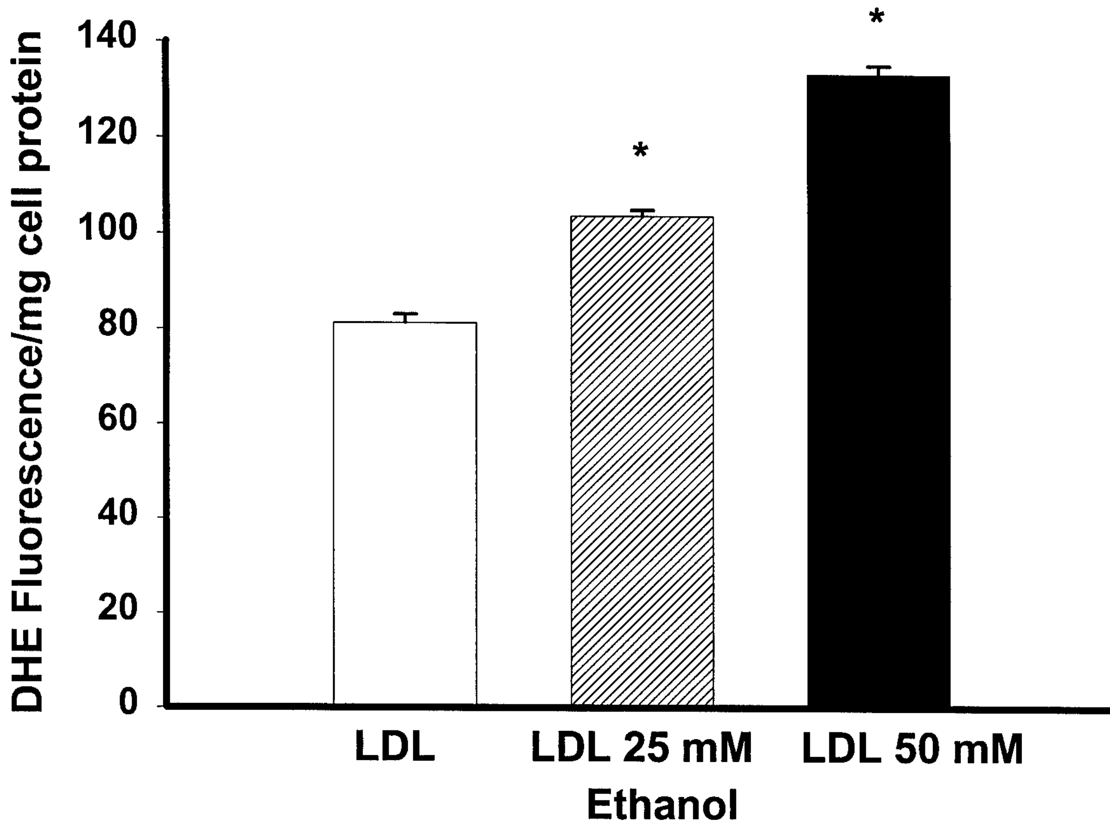
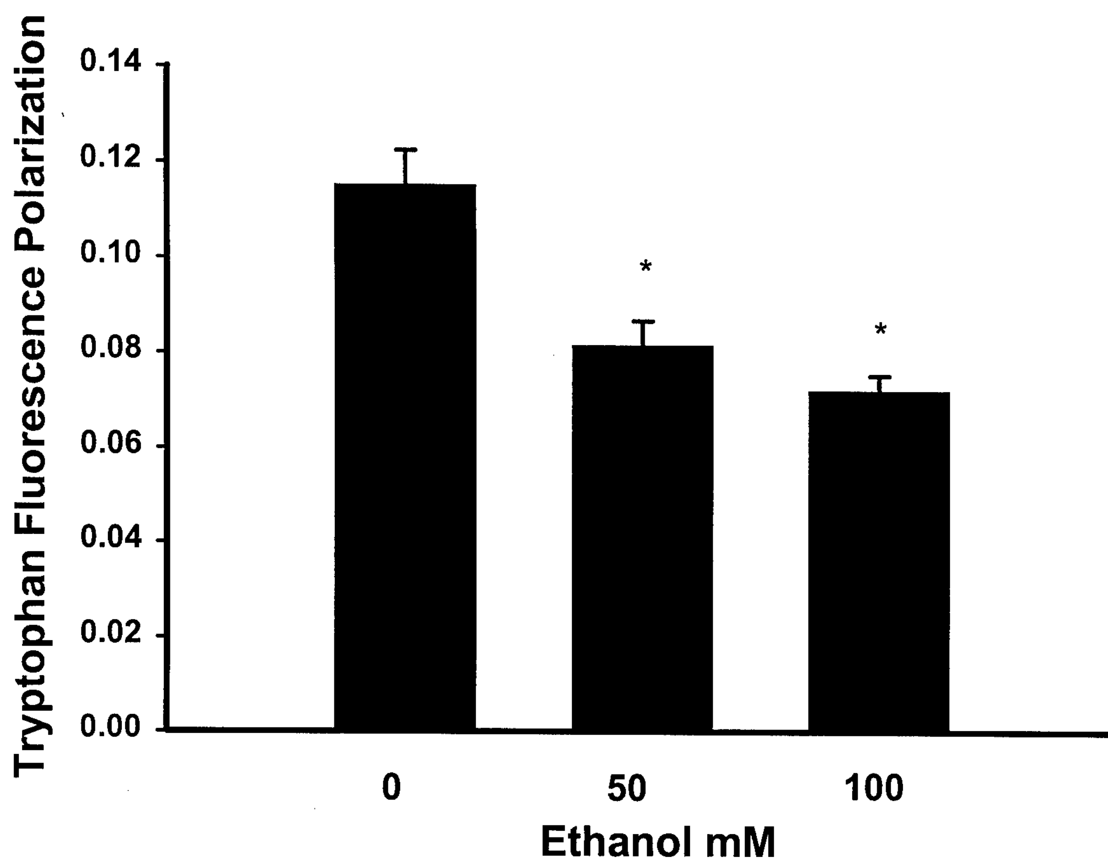


Figure 8. LDL-Mediated DHE Uptake into Cells and Effects of Ethanol



*P ≤ 0.01 as compared to control.

Figure 9. Polarization of Tryptophan Residues of Apolipoprotein B and Effects of Ethanol



*p ≤ 0.001 as compared with 0 ethanol.

Table 1. Cholesterol Distribution in Golgi Complex of Fibroblasts: Effects of Ethanol and Zinc

Treatment	Concentration	% Cholesterol
Control	0 mM	26.23 ± 0.575
Ethanol	40 mM	12.04 ± 0.574*
Ethanol	80 mM	6.30 ± 0.547*
Zinc	25 mM	30.80 ± 1.73*
Ethanol + Zinc	40 mM + 25 mM	15.00 ± 0.337*
Ethanol + Zinc	80 mM + 25 mM	8.89 ± 0.868*

Densitometric analysis of confocal images of fibroblasts labeled with NBD-cholesterol and BODIPY TR ceramide. Data are means ± SEM (n =4-10).

*$P \leq 0.01$ as compared to control.

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Lipid Carrier Proteins and Ethanol

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Key Words

Alcoholism · Apolipoproteins · Cardiovascular disease · Cholesterol · Ethanol · High-density lipoproteins · Lipids · Low-density lipoproteins · Reverse cholesterol transport

Abstract

Ethanol has a pronounced effect on lipid homeostasis. It is our overall hypothesis that certain lipid carrier proteins are targets of acute and chronic ethanol exposure and that perturbation of these proteins induces lipid dysfunction leading to cellular pathophysiology. These proteins include both intracellular proteins and lipoproteins. This paper examines recent data on the interaction of ethanol with these proteins. In addition, new data are presented on the stimulatory effects of ethanol on low-density-lipoprotein (LDL)-mediated cholesterol uptake into fibroblasts and direct perturbation of the LDL apolipoprotein, apolipoprotein B. A cell model is presented that outlines potential mechanisms thought to be involved in ethanol perturbation of cholesterol transport and distribution.

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Lipid homeostasis is altered by chronic ethanol consumption. Ethanol-induced changes in lipid homeostasis have been reported in alcoholics, heavy drinkers and animal models of alcoholism [16, 23, 25, 29, 32]. Examples of ethanol-induced changes in lipid homeostasis are accumulation of triacylglycerols and other lipids in the liver, alterations in cholesterol transport, membrane cholesterol asymmetry, lipoprotein distribution and the polyphosphoinositide cascade. There is increasing evidence that certain lipid carrier or transport proteins including lipoproteins are targets of ethanol [2, 5, 13, 19, 22]. We have been studying the dynamic interaction of ethanol with some of the lipid carrier proteins, and those data are summarized in table 1. Liver fatty-acid-binding protein (L-FABP) is a lipid carrier protein that binds cholesterol, fatty acids, fatty acylcoenzyme A, retinol, heme, hematin, lysophospholipids, bilirubin, prostaglandins and other amphipathic ligands [20]. This protein is found in the liver and other organs. Expression of L-FABP was significantly increased in livers of mice maintained on an ethanol liquid diet for 8 weeks as compared with livers of pair-fed control mice [5]. There was a 43% increase in the amount of L-FABP in livers of the chronic ethanol-treated mice. An earlier study had also found that L-FABP was

Table 1. Lipid carrier proteins and ethanol

Protein	Ethanol dynamics			Ref.
	in vivo	sterol transport	lipid binding	
L-FABP	↑ Liver		No effect C, SA	5, 27
SCP-2	↑ Brain		↓ C, PC, SA	19, 3
Apo A-I			↓ PC, no effect C	4
ApoA-I-PC		↓ Efflux	↓ C	4
HDL		↓ Efflux	No effect C	4
BSA			↓ C, PC, PA	2

C = Cholesterol; PC = phosphatidylcholine; PA = parinaric acid; SA = stearic acid.

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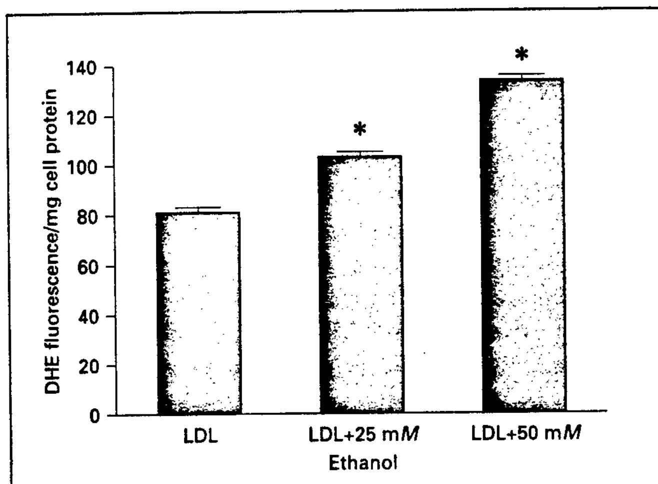
increased in livers of chronic ethanol-treated rats [21]. The increase in L-FABP expression in chronic ethanol-treated animals could be due to ethanol interfering with the binding capacity of L-FABP for its lipid ligands. This potential effect of ethanol on L-FABP was examined using fluorescent-dye-labeled cholesterol and stearic acid. We did not observe any effect of ethanol on cholesterol or stearic acid binding to L-FABP [27]. Chronic ethanol consumption could induce a posttranslational modification of L-FABP that could interfere with lipid binding. In our binding studies, we used rat liver recombinant L-FABP and not L-FABP from livers of chronic ethanol-treated animals. It was reported that the affinity for palmitate was greatly reduced in a delipidated cytosolic liver homogenate of chronic ethanol-treated rats [21]. In the same study, L-FABP amounts increased in the chronic ethanol-treated rats.

Sterol carrier protein 2 (SCP-2) is an intracellular protein that is found in peroxisomes and binds cholesterol, phospholipids and fatty acids [26]. This protein is found in the liver, brain and other organs. The only difference between the liver form of SCP-2 and brain SCP-2 is a single amino acid resulting in a conservative replacement of Ala^{55} for Val^{55} [19]. SCP-2 expression in liver was not affected by chronic ethanol consumption [5]. However, SCP-2 expression was increased in brain tissue of chronic ethanol-treated mice [19]. There was a 56% increase in SCP-2 in synaptosomes of chronic ethanol-treated mice as compared with the pair-fed control mice. In contrast to L-FABP, ethanol inhibited binding of lipids to SCP-2 with cholesterol binding being most affected [3]. The association constants (K_a) of the lipid ligand-SCP-2 complex were in the following order: NBD cholesterol > NBD phosphatidylcholine (PC) > NBD stearic acid. Ethanol, beginning at a concentration of 25 m*M*, significantly reduced the affinity of NBD cholesterol and NBD PC for SCP-2. Effects of ethanol on the K_a of NBD stearic acid were significant only at the highest concentration that was examined (200 m*M*).

The physiological function of SCP-2 has not been elucidated [6, 26]. SCP-2 may be involved in the intracellular trafficking of cholesterol [26]. SCP-2 decreases the half-life of sterol exchange between plasma membranes. SCP-2 is a peroxisomal protein, and transfer of cholesterol to the plasma membrane was found to be dependent on the amount of SCP-2 in peroxisomes [6]. Concomitant with ethanol-induced changes in SCP-2 expression in the brain was the finding that the transbilayer distribution of cholesterol was modified in synaptic plasma membranes of chronic ethanol-treated mice [31]. The amount of cholesterol in the exofacial leaflet was doubled in chronic ethanol-treated mice as compared with control mice. Total cholesterol amounts in synaptic plasma membranes did not differ between the ethanol and control groups. Mechanisms involved in regulating the transbilayer distribution of cholesterol are poorly understood. SCP-2 may be incorporated into the membrane and transports cholesterol from the cytofacial to the exofacial leaflet. Binding of SCP-2 to plasma membranes has been reported [33]. The increased expression of SCP-2 in brains of chronic ethanol-treated mice may be in response to ethanol interfering with binding of ligands to the protein. SCP-2, an intracellular protein, may then be taken up into the cytofacial leaflet that contains approximately 85% of the total synaptic plasma membrane cholesterol and this membrane-bound SCP-2 then translocates cholesterol to the exofacial leaflet. A recent report found that SCP-2 expression inhibited cholesterol efflux from L cell fibroblasts [1].

Cholesterol efflux from cells is an important function whereby cholesterol is removed from cells and transported to the liver. High-density lipoprotein (HDL), lipid-free and lipid-poor apolipoproteins act as acceptors for cholesterol efflux [7, 9, 18, 24, 35]. There has been substantial interest in the relationship between HDL levels and ethanol consumption. A generally accepted finding of both epidemiological and experimental studies is that HDL levels are increased in association with alcohol consumption [8, 11, 12, 14, 15, 23, 28]. Moreover, this lipoprotein and its subfractions are thought to play an important role in the reduced risk of coronary heart disease in moderate alcohol drinkers. What would appear to be an apparent contradiction is that HDL levels have been shown to be elevated in alcoholics but several studies report that the incidence of coronary heart disease is higher in alcoholics even when factors such as cigarette smoking and other factors are taken into consideration [11, 12, 14]. Recent data from our laboratory and another laboratory may contribute to an understanding of this apparent contradiction. We have recently reported that ethanol at concentrations commonly observed during periods of heavy drinking (25 and 50 m*M*) significantly inhibited cholesterol efflux from fibroblasts to HDL and to apolipoprotein A-I (apoA-I)-PC complexes [4]. While ethanol reduced cholesterol efflux to both HDL and apoA-I-PC, the mechanism of action was different. Ethanol inhibited incorporation of cholesterol into apoA-I-PC but did not affect incorporation of cholesterol into HDL. Cholesterol efflux mediated by HDL may result from direct contact of HDL with the cell exofacial leaflet of the plasma membrane, and ethanol may interfere with this contact. ApoA-



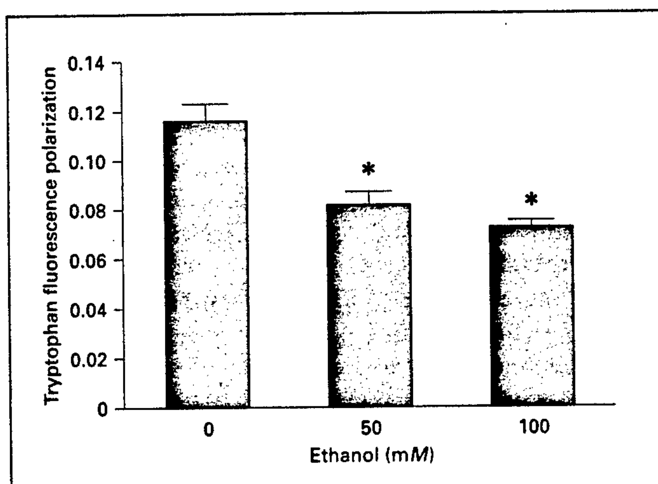


Fig. 1. Effects of ethanol on LDL-mediated cholesterol influx. The fluorescent cholesterol analogue dehydroergosterol (DHE) was incorporated into human plasma LDL. CREF fibroblasts were then incubated with LDL in the presence or absence of 25 and 50 m*M* ethanol for 5 h after which time cells were centrifuged and the fluorescence intensity of dehydroergosterol was measured. Data are means ± SEM of dehydroergosterol in fibroblasts (n = 4 experiments). $^*p \leq 0.005$ as compared to LDL control.

Fig. 2. Ethanol and tryptophan fluorescence polarization of apoB. Steady-state polarization was measured using tryptophan residues of apoB excited at 286 nm and emission at 335 nm in the presence and absence of 50 and 100 m*M* ethanol at 37 °C. Data are means ± SEM of polarization values for apoB tryptophan residues (n = 5 experiments). $^*p \leq 0.001$ as compared to 0 ethanol.

I-PC may incorporate cholesterol by the combined effects of membrane contact and aqueous diffusion. Lakshman's laboratory [22] has shown that HDL of alcoholic patients was less effective in removing cholesterol from mouse macrophages than HDL of control subjects. The uptake of HDL by HepG2 cells was significantly reduced in HDL samples of alcoholic patients. It should be noted that ethanol was not added in that study. A very important conclusion derived from the two studies is that the process whereby cells remove cholesterol is impaired by concentrations of ethanol routinely observed in heavy drinkers and by changes in HDL structure induced by chronic ethanol consumption.

Cholesterol influx mediated by low-density lipoprotein (LDL) is affected by ethanol and is opposite to effects observed for HDL on cholesterol efflux. It can be seen in figure 1 that ethanol significantly increased the uptake of LDL labeled with the fluorescent sterol dehydroergosterol into fibroblasts. There was approximately a 25% increase in fluorescence intensity of fibroblasts incubated with LDL and 25 m*M* ethanol, and 50 m*M* ethanol had even a larger effect on LDL-mediated sterol uptake (fig. 1). Potential mechanisms may include structural changes in apoB, the main apolipoprotein of LDL, providing a more energy-efficient conformation state or changes in the lipid environment associated with the LDL receptor or direct effects on the LDL receptor. We did observe that ethanol decreased fluorescence polarization of tryptophan residues of apoB (fig. 2). On the other hand, fluorescence polarization of tryptophan residues of apoA-I was increased by ethanol [4]. The restricted motion of apoA-I tryptophan residues induced by ethanol may interfere with lipid binding to the apolipoprotein.

Cholesterol transport into and out of cells is altered by ethanol concentrations that routinely occur in heavy drinkers (fig. 3). Ethanol stimulates the uptake of cholesterol into cells and inhibits cholesterol efflux from cells. Several potential mechanisms (LDL receptor binding and expression, LDL protein and lipid domains, selective uptake by coated pits) may explain this increase. Once cholesterol is internalized, it is delivered to lysosomes where the apolipoprotein component of LDL is degraded to amino acids, cholesterol esters are hydrolyzed by acid lipase and cholesterol is transported to different structures. Ethanol could modify the intracellular distribution of cholesterol by affecting the transport to different structures. Ethanol may directly act on HDL or lipid-poor apoA-I and alter protein conformation. Ethanol may perturb the membrane lipid environment that could affect cholesterol efflux. Ethanol could act on acylcoenzyme A cholesterol acyltransferase resulting in changes in the ratio of esterified cholesterol to free cholesterol and such changes affecting reverse cholesterol transport. Ethanol could perturb the Golgi apparatus that has been proposed to be an important component in both transport of cholesterol to the plasma membrane as well as transport from

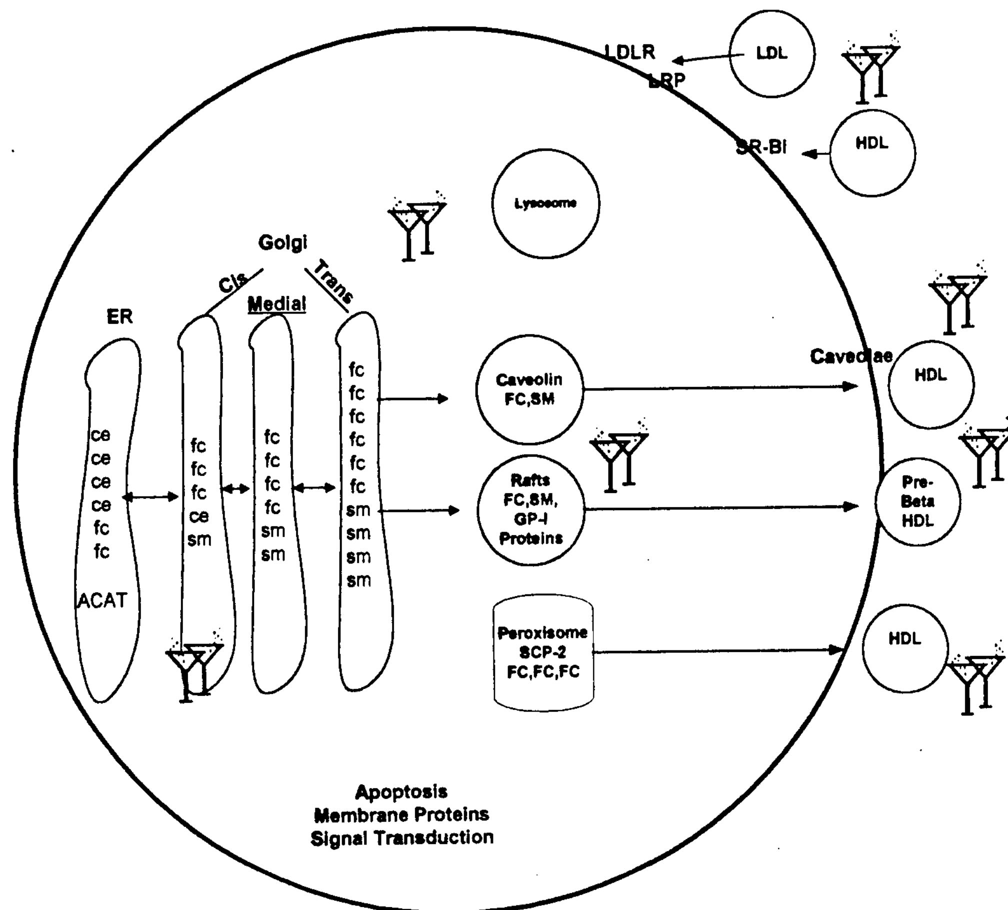


Fig. 3. Potential mechanisms of ethanol effects on cellular cholesterol transport and distribution. Ethanol increases LDL cholesterol uptake by directly altering apolipoprotein structure and possibly the receptor (LDLR). Internalized cholesterol may be processed through an endosome-lysosome pathway and transported to the endoplasmic reticulum (ER) and Golgi apparatus. Ethanol may increase the activity of acylcoenzyme A cholesterol acyltransferase (ACAT) resulting in an increase in esterified cholesterol that would not be available for transport. Transport of cholesterol to the plasma membrane may be inhibited by ethanol acting on caveolin, rafts or SCP-2. Additional mechanisms may include perturbation and structural changes of HDL, pre-β-HDL, caveolae and the scavenger receptor class B type 1 (SR-BI) receptor. The net result of ethanol-induced changes in cellular cholesterol homeostasis may include dysfunction of membrane proteins, the signal transduction pathway leading to apoptosis. ce = Cholesterol ester; fc, FC = free cholesterol; GP-I = glycosylphosphatidyl inositol; LRP = lipoprotein receptor-related protein; sm, SM = sphingomyelin.

the plasma membrane to the Golgi apparatus and other intracellular compartments [9, 17, 24]. Both monensin and brefeldin A inhibited cholesterol efflux to HDL from fibroblasts by acting on the trans-cisternae of the Golgi apparatus and cis- and medial Golgi cisternae, respectively [17]. Agents that disrupt the Golgi apparatus alter the intracellular cholesterol distribution to the plasma membrane and other structures [10]. Chronic ethanol consumption has been shown to alter cholesterol domains in brain plasma membranes, but there are little if any data on intracellular domains and ethanol in any cells [30, 31]. Marked changes in cholesterol distribution can occur in the absence of changes in the total amount of cellular cholesterol that could in turn affect cell function. Lipid transport involving caveolin, vesicles and SCP-2 to the plasma membrane could be altered by ethanol.

One conclusion is that cells of individuals who consume at least 5–6 drinks contain more cholesterol as compared to moderate drinkers. Therefore, cholesterol homeostasis of heavy drinkers is impacted by the acute and chronic effects of ethanol. It is well established that cholesterol plays an important role in cell structure and func-

tion. Membrane fluidity and activity of Na^+-K^+-ATPase are regulated by cholesterol. Apoptosis was induced by loading macrophages with cholesterol, and this lethal effect of cholesterol was thought to be due to activation of the Fas pathway [34]. Acute and chronic effects of ethanol on cellular cholesterol homeostasis could certainly contribute to pathophysiology occurring in alcoholics.

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Ethanol inhibits cholesterol efflux from cells to HDL and apolipoprotein-AI-PC complexes (<I>Biochemistry</I>,39, 10599-10606,2000). The Golgi complex is involved in regulation of cholesterol efflux and ethanol may interfere with this regulation. We examined effects of ethanol on cholesterol content of the Golgi complex in NIH 3T3 fibroblasts using confocal microscopy and densitometric analysis. The fluorescent cholesterol analogue, NBD-cholesterol and the fluorescent labeled probe for Golgi, BODIPY TR ceramide were used. Ethanol modified cholesterol content in Golgi but the direction of effects was concentration dependent. Ethanol at a concentration of 40 mM significantly increased cholesterol in the Golgi complex however, 80 mM ethanol significantly decreased cholesterol content. Zinc, that previously has been shown to stimulate cholesterol transport, significantly decreased cholesterol content in the Golgi complex and abolished the stimulatory effects of 40 mM ethanol. Reduction of cholesterol by 80 mM ethanol was furthered increased by zinc. Effects of 40 mM ethanol on cholesterol content of Golgi are consistent with our data showing that 25 and 50 mM ethanol inhibited cholesterol efflux from cells to HDL and apoA-I-PC complexes. Ethanol could disrupt formation of lipid rafts in the Golgi complex resulting in an accumulation of cholesterol. Alternatively, ethanol could stimulate transport of cholesterol from other organelles to the Golgi complex. Both 80 mM ethanol and zinc singularly and combined reduced cholesterol in Golgi. It is unclear if the mechanisms for those effects of ethanol and zinc are the same or are different. Ethanol modifies cholesterol homeostasis in the Golgi complex and such modification could certainly could impact on lipid and protein sorting and sphingomyelin synthesis. This work was supported by the US Army Medical Research and Material Command (DAMD17-00-1-0583).