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13. ABSTRACT (Maximum 200 Words)

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. An overriding conclusion of the work of the past 3 years is that ethanol at concentrations observed in problem drinkers and alcoholics (e.g., an individual consuming either 6 beers, or 6 one shots of whiskey, or 6 glasses of wine) has a profound and multifaceted effect on cellular regulation of cholesterol. Our work to date shows that many of the systems involved in regulating cholesterol transport are perturbed by ethanol (HDL, apoA-I, LDL, PC-PLC, PC-PLD). The Golgi complex plays an important role in protein and lipid trafficking and our new data confirm that the Golgi complex is perturbed by ethanol. Moreover, we are now able to measure the distribution of cholesterol in the Golgi complex and we now show that ethanol has a very specific effect on regions of the Golgi complex and acts to reduce cholesterol in the trans-Golgi region.

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TITLE: Mechanisms of Alcohol Induced Effects on Cellular Cholesterol Dynamics

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ABBREVIATIONS

ApoA-I, apolipoprotein A-I

CHD, coronary heart disease

DHE, dehydroergosterol

G1, gradient fraction 1

G2, gradient fraction 2

G3, gradient fraction 3

HASMC, human aortic smooth muscle cells

HBBS, HEPES buffer saline solution

HCAEC, human coronary artery endothelial cells

HDL, high density lipoprotein

LDL, low density lipoprotein

PA, phosphatidic acid

PC-PLC, phosphatidylcholine-phospholipase C

PC-PLD, phosphatidylcholine-phospholipase D

Petoh, phosphatidylethanol

TNS, trypsin neutralizing solution

INTRODUCTION

Risk of coronary heart disease (CHD) morbidity and mortality is reduced by moderate alcohol consumption whereas some studies suggest that heavy alcohol consumption has little or no benefit for CHD[1-4]. A generally accepted finding of epidemiological and experimental studies is that high-density lipoproteins (HDL) levels are increased in association with alcohol consumption [3;5-7]. An increase in HDL has been proposed to be one of the potential factors involved in the protective effects of moderate alcohol consumption on CHD [5;8]. HDL levels however, are increased in heavy drinkers but their risk of CHD is higher than that of moderate alcohol drinkers [9-11]. An important function of HDL is removal of excess lipids including cholesterol from cells and evidence has shown that chronic and acute administration of ethanol alters lipoprotein-mediated cholesterol efflux. Cholesterol efflux was decreased in mouse macrophages incubated with HDL of human alcoholic subjects [12]. However, it was recently reported that cholesterol efflux was stimulated from Fu5AH rat hepatoma cells to plasma of human subjects who consumed 40 g of alcohol per day for 3 weeks [13]. Acute administration of a very high ethanol concentration (350 mM) increased the rate of cholesterol efflux from erythrocytes to plasma [14]. Ethanol concentrations (25 and 50 mM) that are seen in alcoholics and heavy drinkers inhibited cholesterol efflux from rat fibroblasts to HDL and apoA-I [15]. 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 such as phosphatidylcholine-phospholipase C (PC-PLC) and phosphatidylcholine-phospholipase D (PC-PLD) that are involved in reverse cholesterol transport. These potential mechanisms are being studied in this grant that consists 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 and human endothelial cells are being used in the experiments.

BODY OF REPORT

Ethanol Reduces Cholesterol in the Golgi Complex of Human Aortic Smooth Muscle Cells (HASMC). We had previously shown that ethanol inhibited cholesterol efflux from HASMC to HDL and ApoA-1 that was part of Objective 3 of the grant. The Golgi complex plays an important role in cholesterol trafficking [16] and we proposed that ethanol may act at the level of the Golgi complex and this was part of both Objectives 2 and 3. Effects of cholesterol levels on the Golgi complex were determined using fluorescent probes for cholesterol and the Golgi complex and confocal microscopy. HASMC were incubated with NBD-cholesterol that is a fluorescent-labeled sterol for 2.5 h. Cells were then incubated with BODIPY-ceramide that is a

fluorescent marker for the Golgi complex. Panels A, D, G, J and B, E, K, and H show fluorescence of NBD-cholesterol and BODIPY ceramide in HASMC, respectively. Panels C, F, I, and L show co-localization of the two fluorescent probes. Co-localization was reduced by ethanol treatment and percent changes in co-localization are shown in Figure 2.

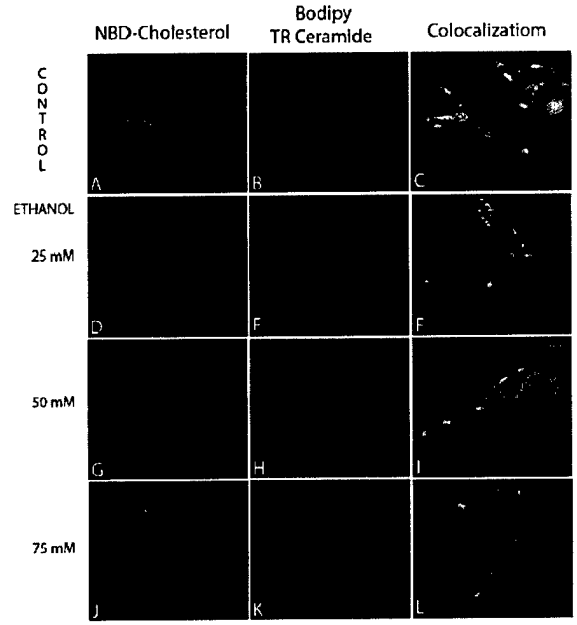

Figure 1. Confocal microscopy of HASMC incubated with ethanol.

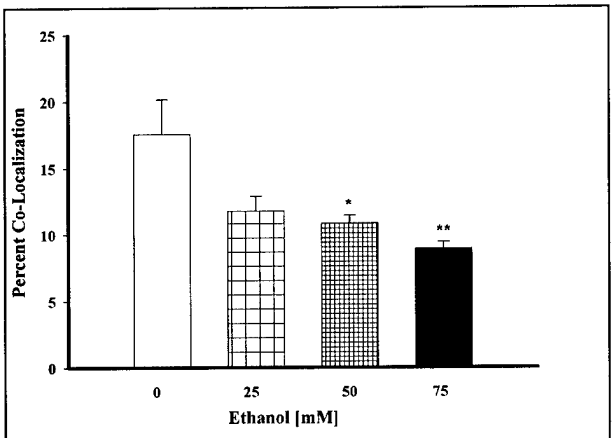

Figure 2. Effects of Ethanol on Percent Co-Localization of NBD-Cholesterol and BODIPY TR Ceramide in HASMC. Cells were incubated with ethanol concentrations for 2 h. Perecnt Co-localization was determined using MetaMorph software and data are means ± SEM (n=9).
*$p \leq 0.02$; *$p \leq 0.004$.

Significantly less co-localization occurred at 50 and 75 mM ethanol. There was a reduction in co-localization at 25 mM ethanol but this difference was not significantly different from the control cells. Changes in co-localization are indicative of a change in cholesterol levels. Ethanol reduced co-localization and this was interpreted as a reduction in cholesterol in the Golgi complex.

Cholesterol Distribution in the *Trans* and *Cis*-Medial Regions of the Golgi Complex and Effects of Ethanol in Human Aortic Smooth Muscle Cells. Data in Figures 1 and 2 were indicative of ethanol reducing cholesterol in the Golgi complex as revealed by confocal microscopy and the fluorescent cholesterol analogue, NBD-cholesterol and a fluorescent marker for the Golgi complex, BODIPY TR ceramide [17]. The Golgi complex consists of different regions and it has been reported that there is an increasing gradient of cholesterol from the *cis* region to the *trans* region of the Golgi complex [18]. We have applied a method in our lab to isolate different Golgi regions using sucrose density centrifugation. Our initial studies were with an immortalized astrocyte cell line because those cells are provided to our lab at little or no costs as compared with the relatively expensive HASMC and those studies were reported in the previous annual report and a recent paper [19]. HASMC were harvested and washed twice with PBS. The cells were suspended in G-buffer (10 mM Tris-HCl, 0.25 M Sucrose and 2 mM $MgCl_2$, pH 7.4) containing 10 mM $CaCl_2$. Leupeptin and PMSF were added to the cell suspension to inhibit proteolytic enzymes, prior to homogenization. The cells were homogenized in a Potter-type homogenizer and homogenized with 20-30 strokes using a serrated homogenizing pestle. The homogenate was centrifuged at 5,000 x g for 10 min. The pellet was suspended and

homogenized in 1.4 M sucrose and over layered with 0.8 M, 1.0 M, 1.2 M sucrose gradients in ultracentrifuge tubes. Samples were then centrifuged at 95,000 x g for 2.5 h in a SW28 rotor in a Beckman L8-70 ultracentrifuge. Each interface was carefully removed and diluted with G buffer and centrifuged in a SS34 rotor at 45,900 x g for 20 min. Each pellet was suspended in buffer and used for the experiments. Protein concentrations of the fractions were determined using Bradford assay. Cholesterol content in each fraction was determined enzymatically in a microassay using the Boehringer-Mannheim diagnostic kit [20] and procedures reported by our laboratory [21-23].

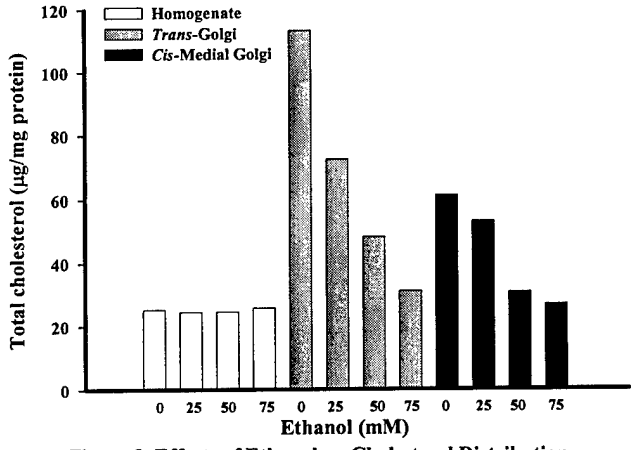

Figure 3. Effects of Ethanol on Cholesterol Distribution in the Golgi complex of HASMC. Cells were incubated with different concentrations of ethanol for 2 h.Golgi was isolated as described in the text.

Figure 3 shows that the two fractions thought to represent the *trans* Golgi and the *cis*-medial region contained different levels of cholesterol. Cholesterol abundance of the *trans*-Golgi region was approximately two-fold higher compared with the *cis*-medial Golgi region. These results are similar to other cell types in which cholesterol levels were higher in the *trans*-Golgi as compared with the *cis*-medial region [18;19]. It can be seen in Figure 3 that ethanol reduced cholesterol levels in a concentration dependent manner in the *trans*-Golgi region. Effects of ethanol on the cis-medial fraction were not as straightforward as observed with the *trans*-Golgi region. Both 50 mM and 75 mM ethanol reduced cholesterol levels but there was only a marginal effect at 25 mM ethanol. Effects of ethanol on Golgi cholesterol levels cannot be explained by differences in synthesis or degradation because cholesterol levels in the cell homogenate fractions did not differ (Figure 3). We are presently repeating these experiments but it does appear that ethanol has a greater effect on cholesterol levels in the *trans*-Golgi region than the *cis*-medial region. Ethanol appears to induce movement of cholesterol from the Golgi complex. Destination of this cholesterol is not known and this will be examined in future experiments.

Phosphatidylcholine-Phospholipase D (PC-PLD) Activity in Human Aortic Smooth Cells Incubated with ApoA-1. PC-PLD is associated with the Golgi complex and this enzyme is thought to play a role in cholesterol efflux.We showed in the previous annual report that ethanol stimulated activity of PC-PLD. However, those experiments were accomplished in the absence of apoA-1 or HDL that are thought to act on PC-PLD by a G-protein coupled

receptor. To begin to understand activation of PC-PLD, we incubated HASMC with apoA-1 for different time periods (15, 30, 60, 120 min). Cells were in a lipoprotein-deficient medium. PC-PLD activity was quantified using an enzyme immunoassay kit. Figure 4 shows that PC-PLD activity was significantly reduced when incubated with ApoA-1 and 15, 30 and 60 minutes as compared with the control condition where apoA-1 was not present. Greater inhibition was observed at the earlier time points and activity returned to control levels after 120 min incubation in the presence of apoA-1. One interpretation of the data is that abundance of PC-PLD is being reduced by its action of hydrolyzing phosphatidylcholine. Maximum effects of apoA-1 on PC-PLD were observed at after the 15 min incubation with apoA-1 compared with the longer incubation times. However, it is quite possible that a greater reduction in PC-PLD activity may occur earlier than 15 min. The kinetics of this reaction will be examined in future experiments. Moreover, effects of ethanol on PC-PLD activity in the presence of both apoA-1 and HDL will be determined. As mentioned, ethanol increased PC-PLD activity but the experiment was done in the absence of apoA-1 or HDL.

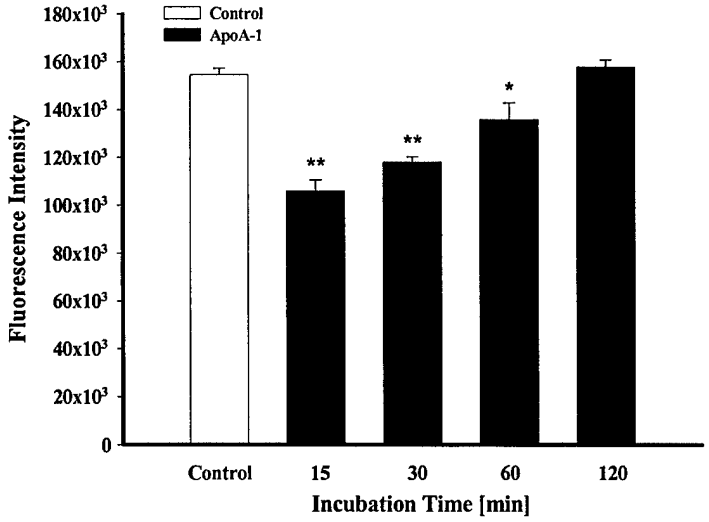

Figure 4. PC-PLD Activity in Human Aortic Smooth Muscle Cells Incubated with ApoA-1. Cells were incubated with apoA-1 for different time periods. PC-PLD activity was determined using an enzyme immunoassay kit. Data are means ± SEM (n=4). *p ≤ 0.05; **p ≤ 0.001

Effects of Ethanol and Incubation Time on LDL Uptake in Human Coronary Artery Endothelial Cells (HCAEC). We have found that ethanol at concentrations observed in alcoholics and heavy drinkers inhibited cholesterol efflux to HDL and apoA-I in cells. Preliminary data in the previous report revealed that ethanol stimulated uptake of LDL in HCAEC. The purpose of the experiments reported below was to replicate our findings and complete that portion of the proposed experiments. HCAEC were incubated with fluorescent labeled LDL and ethanol for 2.5 and 5.0 h. Fluorescence intensity was quantified by measuring intensity in a fluorescence microplate reader.

Data in Figure 5 show that the only significant effect of ethanol was at a concentration of 25 mM when cells were incubated with LDL for 5 h. In the previous report we showed that ethanol increased LDL uptake not only at 25 mM but also at 50 and 75 mM at 5 h but not 2.5 h of incubation. Effects of ethanol were similar for each concentration. Incubation with LDL for 5 h significantly increased LDL uptake and those findings are in agreement with what we had previously observed. We were able to replicate some but not all of the previous findings. The earlier data were based on an N of 1 and data in Figure 5 is from 3 separate experiments. Quality of the fluorescent labeled LDL is not a factor. The 5 h incubation time resulted in greater uptake in both sets of experiments. One procedural difference was that in the earlier experiment fluorescence was measured in a fluorimeter a single sample at a time. In the current work, a fluorescence microplate reader was used enabling all samples to be read at the same time. Our data indicate that LDL uptake appears to be increased by ethanol. We will use other method to verify our results. Cells will be incubated with radiolabeled LDL and effects of ethanol determined. This technique will eliminate any potential artefacts arising from using fluorescent labeled LDL.

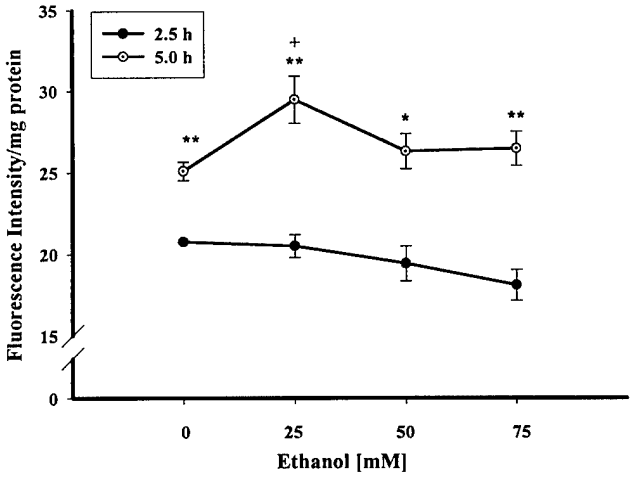

Figure 5. Effects of Ethanol on Uptake of LDL in Human Coronary Artery Endothelial Cells (HCAEC). Cells were incubated with BODIPY-LDL and different concentrations of ethanol for 2.5 h and 5.0 h. Fluoresence intensity was deternmined using a fluorescence plate reader. Data are means ±SEM. *p ≤ 0.01; **p ≤ 0.005 as compared to 2.5 h. +p ≤ 0.05 as compared with 0 mM ethanol at 5 h.

The effects of 25 mM ethanol are intriguing. We had observed such an effect in the earlier work and thought that perhaps lower ethanol concentrations may be effective in increasing LDL uptake that could be viewed as beneficial. We tested ethanol concentrations of 7.5 mM 10 mM and 15 mM. LDL uptake was not altered by lower ethanol concentrations (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

- Ethanol reduces cholesterol levels in the Golgi complex of human aortic smooth muscle cells as revealed by confocal microscopy.
- Using density gradient centrifugation to separate the Golgi complex regions, we found that ethanol alters cholesterol distribution within the Golgi complex of human aortic smooth muscle cells. The trans-Golgi region was more susceptible to effects of ethanol compared with the *cis*-medial-Golgi region.
- PC-PLD activity was reduced in human aortic smooth muscle cells when incubated with apoA-1.
- A low concentration of ethanol (25 mM) increased LDL uptake into human coronary artery endothelial cells. Lower ethanol concentrations (7.5-15 mM) did not alter LDL uptake.

REPORTABLE OUTCOMES

Igbavboa, U., Pidcock, J.M., Malo, T.M., Studniski, A.E., Johnson, L.N.A., Wood, W.G. HDL and apolipoprotein A-I induced cholesterol efflux is inhibited by ethanol in human smooth muscle aortic cells: Roles of phospholipases D and the Golgi complex. *Alcohol and Alcoholism*, 38, 474, 2003.

Igbavboa, U., Pidcock, J.M., Malo, T.M., Johnson, L.N.A., Wood, W.G. Cholesterol efflux is inhibited by ethanol in human aortic smooth muscle cells: Differences in sensitivity of HDL and apolipoprotein A-I and the roles of phosphatidylcholine-phospholipase C and D. *Metabolism*, 2003, submitted.

Igbavboa, U., Pidcock, J.M., Jognson, L.N.A., Malo, T.M., Sun, G.Y., and Wood, W.G. Cholesterol distribution in the Golgi complex of DITNC1 astrocytes is differentially altered by fresh and amyloid β-peptide$_{1-42}$. *Journal of Biological Chemistry*, 278, 17150-17157, 2003.

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. An overriding conclusion of the work of the past 3years is that ethanol at concentrations observed in problem drinkers and alcoholics (e.g., an individual consuming either 6 beers, or 6 one shots of whiskey, or 6 glasses of wine) has a profound and multifaceted effect on cellular regulation of cholesterol. Our work to date shows that many of the systems involved in regulating cholesterol transport are perturbed by ethanol (HDL, apoA-I, LDL, PC-PLC, PC-PLD). The Golgi complex plays an important role in protein and lipid trafficking and our new data confirm that the Golgi complex is perturbed by ethanol. Moreover, we are now able to measure the distribution of cholesterol in the Golgi complex and we now show that ethanol has a very specific effect on regions of the Golgi complex and acts to reduce cholesterol in the *trans*-Golgi region.

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Cholesterol Efflux is Inhibited by Ethanol in Human Aortic Smooth Muscle Cells: Differences in Sensitivity of HDL and Apolipoprotein A-I and the Roles of Phosphatidylcholine-Phospholipase C and D

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ABSTRACT

Background: Moderate alcohol consumption is associated with a reduced risk of cardiovascular morbidity and mortality. An increase in high-density lipoproteins (HDL) that are involved in cholesterol efflux are one of several factors that are thought to contribute to this reduced risk. However, HDL levels are elevated in heavy drinkers and alcoholics but their risk is not reduced. Ethanol may interfere with cholesterol efflux from cells and experiments in this paper report on acute effects of ethanol on cholesterol efflux from human aortic smooth muscle cells (HASMC) to HDL, apolipoprotein A-I (apoA-I) and the roles of phosphatidylcholine-phospholipase C (PC-PLC) and PC-phospholipase D (PC-PLD). Both PC-PLC and PC-PLD are involved in cholesterol efflux and also acted upon by ethanol. **Method:** Cholesterol efflux to HDL and apoA-I was determined in HSMAC using the fluorescent cholesterol analogue, dehydroergosterol. PC-PLC and PC-PLD activities were determined using an enzyme coupled assay. **Results:** Ethanol significantly inhibited HDL-mediated cholesterol efflux from HASMC. Significant effects of ethanol were seen at ethanol concentrations of 50 and 75 mM but not at 25 mM ethanol. ApoA-I was more effective in inducing sterol efflux than was HDL and significant inhibition occurred beginning at 25 mM ethanol. Ethanol stimulated activity of PC-PLD and PC-PLC but effects of ethanol were greater on PC-PLD. **Conclusions:** Ethanol may promote movement of cholesterol to the plasma membrane by stimulating phospholipase C and D-induced vesicular transport. However, removal of cholesterol from the plasma membrane may be inhibited by ethanol interfering with the physico-chemical interaction of HDL and particularly apoA-I with cell surface proteins. Heavy drinkers and alcoholics may be at risk with respect to acute and chronic effects of ethanol on cholesterol efflux and conversion of lipid-free apoA-I to mature HDL.

Keywords: Apolipoprotein A-I; Cholesterol Efflux; HDL; Human Aortic Smooth Muscle Cells; Phosphatidylcholine-Phospholipase C; Phosphatidylcholine-Phospholipase D

Running title: Ethanol Inhibits Cholesterol Efflux in Human Aortic Cells

INTRODUCTION

Risk of coronary heart disease (CHD) morbidity and mortality is reduced by moderate alcohol consumption whereas some studies suggest that heavy alcohol consumption has little or no benefit for CHD (Eagles and Martin, 1998; Klatsky, 1999; Rimm, et al., 1999; Sesso and Gaziano, 1999). A generally accepted finding of epidemiological and experimental studies is that high-density lipoproteins (HDL) levels are increased in association with alcohol consumption (Castelli, et al., 1977; Klatsky, 1994; Rimm, et al., 1999; Srivastava, et al., 1994). An increase in HDL has been proposed to be one of the potential factors involved in the protective effects of moderate alcohol consumption on CHD (Castelli, et al., 1977; Criqui, et al., 1987). HDL levels however, are increased in heavy drinkers but their risk of CHD is higher than that of moderate alcohol drinkers (Frohlich, 1996; Goldberg, et al., 1995; Hojnacki, 1994). An important function of HDL is removal of excess lipids including cholesterol from cells and evidence has shown that chronic and acute administration of ethanol alters lipoprotein-mediated cholesterol efflux. Cholesterol efflux was decreased in mouse macrophages incubated with HDL of human alcoholic subjects (Rao, et al., 2000). However, it was recently reported that cholesterol efflux was stimulated from Fu5AH rat hepatoma cells to plasma of human subjects who consumed 40 g of alcohol per day for 3 weeks (van der Gaag, et al., 2001). Acute administration of ethanol (350 mM) increased the rate of cholesterol efflux from erythrocytes to plasma (Daniels and Goldstein, 1982). Ethanol concentrations (25 and 50 mM) that are seen in alcoholics and heavy drinkers inhibited cholesterol efflux from rat fibroblasts to HDL and apoA-I (Avdulov, et al., 2000).

Removal of lipids from cells involves several different mechanisms (e.g., membrane receptors, lipoproteins, signaling pathways, Golgi complex) that could be targets of ethanol

perturbation. Ethanol for example has been reported to have a greater effect on lipid incorporation into apoA-I as compared with HDL (Avdulov, et al., 2000). ApoA-I is thought to activate phosphatidylcholine-PLD (PC-PLD) and phosphatidylcholine-phospholipase C (PC-PLC) resulting in phospholipid hydrolysis and production of phosphatidic acid (PA) and diacylglycerol, respectively (Walter, et al., 1996). Both PC-PLC and PC-PLD appear to be two of the mechanisms involved in regulating cholesterol efflux from cells (Walter, et al., 1996). PC-PLD is of particular importance with respect to action of ethanol because in the presence of alcohols there is a transphosphatidylation reaction and a phosphatidylalcohol is formed and a reduction of PA production (Heller, 1978; Moehren, et al., 1994). PA has been shown to stimulate cholesterol efflux (Walter, et al., 1996). Ethanol may alter activity of PC-PLC and PC-PLD that could impact on cholesterol efflux. Activity of PC-PLC in hepatocytes and activity of PC-PLD in fibroblasts were enhanced by ethanol (Kiss and Anderson, 1990;Pittner and Fain, 1992). Butanol which has a membrane/buffer partition coefficient several orders of magnitude higher than ethanol, inhibited cholesterol efflux of human fibroblasts and reduced production of phosphatidic acid (Walter, et al., 1996). In the same study it was shown that inhibition of PC-PLC by D 609 reduced cholesterol efflux and it was concluded that both PC-PLD and PC-PLC play a role in cholesterol efflux (Walter, et al., 1996).

The purpose of the experiments reported in this paper were to determine if acute ethanol administration altered cholesterol efflux induced by HDL and apoA-I in cells that are directly involved in cardiovascular function. Human aortic smooth muscle cells (HASMC) were used in this study. ApoA-I has been proposed to interact with the ATP binding cassette transporter 1 (ABC1) to remove cholesterol in contrast to HDL and HASMC are enriched in ABC1 (Fielding, et al., 2000; Wang, et al., 2000). Cholesterol efflux mediated by HDL and apoA-I may differ in

response to ethanol. In addition, effects of ethanol on activation of PC-PLC and PC-PLD activity were determined in HASMC using an enzyme coupled assay and *N*-acetyl-3,7-dihydroxyyphenoxazine (Zhou, et al., 1997).

METHODS

Materials. ApoA-I and HDL were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Amplex Red phosphatidylcholine-specific phospholipase C (A-12218) and phospholipase D (A-12219) assays kits were obtained from Molecular Probes (Eugene, Oregon). Human aortic smooth muscle cells and cell growth media (Smooth Muscle Cell Basal Medium (SmBM®), growth factors, trypsin/EDTA , HEPES Buffer Saline Solution (HBBS) and Trypsin Neutralizing Solution (TNS) were all obtained from Bio*Whittaker Company (Walkersville, MD). FBS and antimytotic were obtained from HyClone, (Logan,UT). Lipoprotein deficient serum from human plasma was obtained from Sigma-Aldrich Chemicals (St. Louis, MO). 96-well plates for were purchased from USA Scientific, (Woodland, CA). L-Glutamine was obtained from Invitrogen:GibCo Corp. (Carlsbad, CA). Ethanol and all other reagents and chemicals were obtained from Sigma-Aldrich Chemicals (St. Louis, MO).

Cell Culture. Human aortic smooth muscle cells (HASMC) were grown in 25 cm^2 flask using SmBM® media and SMGM®- 2 Singlequot ®. The flasks with cells were maintained in an incubator at 37°C with 5% CO_2 . Media was replaced every 2 days. All experiments were performed with confluent cells.

Cholesterol Efflux. Confluent HASMC were preloaded with the fluorescent cholesterol analogue, dehydroergosterol (DHE) (3 μg/ml of cell culture medium) for 18 h in 0.5% lipoprotein deficient serum. DHE incubation time and concentration were determined from sampling incorporation of DHE into HASMC over 0-24 h. The optimal HDL or apoA-I concentration for our experiment was obtained by incubating the cells with different concentrations of HDL (10-40 μg of protein/ml of cell culture medium) or apoA-I for 2 h. HDL (30 μg of protein/ml of cell culture medium) was used in the cholesterol efflux experiments. In

experiments using apoA-I, 30 μg of apoA-I/ml of cell culture medium were used and this amount is based on our recent report (Avdulov, et al., 2000). Cells were incubated for a period of 30 min with 0, 25, 50, or 75 mM ethanol after which 30 μg of human plasma HDL protein/ml of medium or 30 μg of apoA-I/ml of medium was added and cells were further incubated for 2 h. Cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer. The protein of the homogenized samples was determined by Bradford method. Sample (25μg protein) was placed in a quartz cuvette and DHE fluorescence intensity determined using a LS-50B fluorimeter (Perkin-Elmer, Norwalk, CT) with the excitation wavelength of 324 nm and emission wavelength of 376 nm.

PC-PLC and PC-PLD Assays. PC-PLC activity and PC-PLD activity in HASMC were quantified using the Amplex red PC-PLC and PC-PLD assay kits (Molecular Probes, Eugene, OR). Cells were incubated with ethanol (0, 25, 50, and 75 mM) for 2 h after which time cells were then washed twice with HBBS, trypsinized and neutralized with TNS or serum, collected, and centrifuged at 14,000 rpm for 1 min in an Eppendorf centrifuge (model 541 7R). The pellet containing cells was washed twice with 1 ml PBS and homogenized in a loose-fitting homogenizer and aliquots placed in 96 well-plates and the assay reagents added. The intensity of the Amplex Red complex formed was measured after 60 min in a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Device, Sunnyvale, CA) using an excitation wavelength of 542 nm and an emission wavelength of 590 nm.

RESULTS

Incorporation of Dehydroergosterol in HASMC. DHE is a sterol occurring in yeast, it is very similar to cholesterol in its structure and function, and it has been previously used as a cholesterol analogue in several different studies of sterol dynamics (reviewed in: Mukherjee, et al., 1998; Schroeder, et al., 1996). DHE is an ideal cholesterol analogue because the sterol is naturally fluorescent containing an endogenous fluorescent conjugated triene system in the B and C rings. DHE transport kinetics are analogous to those of cholesterol (Kier, et al., 1986; Mukherjee, 1998; Nemecz, et al., 1988). The purpose of these baseline experiments was to determine optimal conditions for DHE incorporation into HASMC. Three different concentrations of DHE (1,2,3 μg/ml of media) were incubated with HASMC for 18 h. Incorporation of DHE into cells was monitored by an increase in DHE fluorescence intensity. Figure 1 shows that DHE concentration of 3 μg/ml of media produced a robust increase in fluorescence intensity compared to the two lower concentrations. Fluorescence of non-incorporated DHE in the medium was negligible. The magnitude of fluorescence intensity was dependent also on the duration of incubation of DHE with cells. Fluorescence intensity of DHE steadily increased over time plateau ng between 18 and 24 h of incubation (Figure 2).

Sterol Efflux to HDL and ApoA-I. Figure 3 shows the effects of increasing concentrations of HDL on sterol efflux from HASMC. DHE fluorescence was measured in cells following removal of HDL by centrifugation. HDL significantly removed DHE from HASMC, and this effect was maximal at 30 μg of HDL protein/ml of medium and did not change significantly when 40 μg of HDL protein/ml of medium was added (Figure 3).

There was approximately a 20% reduction in cholesterol in cells incubated with HDL alone compared with no HDL and ethanol significantly inhibited HDL-mediated cholesterol

efflux from HASMC (Figure 4). Significant effects of ethanol were seen at ethanol concentrations of 50 and 75 mM ($p < 0.04$) but not at 25 mM ethanol (Figure 4). Effects of ethanol at 50 and 75 mM resulted in approximately 8-10% of cholesterol being removed from cells by HDL as compared to 20% removal by HDL alone.

There was a 24% reduction in sterol in cells when incubated with apoA-I alone as compared with cells not incubated with apoA-I (Figure 5). Ethanol significantly ($p \leq 0.001$) inhibited sterol efflux beginning at a concentration of 25 mM ethanol (Figure 5). In the presence of 50 and 75 mM ethanol, there was only approximately 6% and 3% of sterol removed respectively, by apoA-I.

PC-PLD and PC-PLC Activity. Both PC-PLC and PC-PLD have been proposed to be involved in cholesterol efflux. PC-PLD was significantly stimulated by ethanol (Figure 5). There was a 2-fold increase in PC-PLD activity by 25 mM ethanol as compared to control cells. It can be seen in Figure 5 that both 50 and 75 mM ethanol significantly ($p \leq 0.001$) increased PC-PLD activity as compared to control cells but this effect was not as large as observed for 25 mM ethanol.

Activity of PC-PLC was significantly ($p \leq 0.03$) increased by 25 mM ethanol as compared to the control cells resulting in a 16% increase in activity of the enzyme (Figure 6). PC-PLC activity was higher in the presence of 50 or 75 mM ethanol as compared to control but these differences were not significant.

DISCUSSION

Experiments in this paper tested the hypothesis that ethanol at concentrations observed in heavy drinkers and alcoholics would alter cholesterol efflux induced by HDL and apoA-I in human aortic smooth muscle cells. ApoA-I was used because recent evidence suggest that apoA-I may directly interact with cell surface receptors and that ethanol has a differential effect on apoA-I as compared with HDL (Fielding, et al., 2000; Avdulov, et al., 2000). In addition, effects of ethanol on PC-PLC activity and PC-PLD activity were examined. Both phospholipases have been previously shown to be involved in regulation of cholesterol efflux and acted upon by ethanol. We found that acute administration of ethanol inhibited cholesterol efflux from human aortic smooth muscle cells to HDL and apoA-I. Cholesterol efflux induced by apoA-I was more sensitive to effects of ethanol as compared with HDL. PC-PLD activity and PC-PLC activity were increased by ethanol with the greatest effect seen on PC-PLD activity.

Acute administration of ethanol inhibited cholesterol efflux induced by HDL and apoA-I from human aortic smooth muscle cells. ApoA-I had a greater effect on cholesterol efflux and was more sensitive to effects of ethanol compared with HDL. Significant inhibition of cholesterol efflux occurred at an ethanol concentration of 25 mM in the presence of apoA-I versus 50 mM in the presence of HDL. Differences in the magnitude of effects of ethanol on HDL and apoA-I may be due to direct effects of ethanol on those structures and interaction with cell surface proteins. Previously it has been reported that ethanol inhibited incorporation of cholesterol into apoA-I-PC complexes but did not alter incorporation of cholesterol into HDL (Avdulov, et al., 2000). There is also evidence that lipid efflux to apoA-I involves proteins at the cell surface such as ABC-1 transporter and caveolin (Fielding, et al., 2000; Lawn, et al., 1999). Recently, it was reported that cholesterol efflux from HASMC to apoA-I was derived from

membrane microdomains enriched in caveolin (Fielding, et al., 2002). Ethanol has been reported to decrease the level of caveolin in astrocytes (Megias, et al., 2000) that might interfere with lipid efflux.

PC-PLC and PC-PLD have been shown to contribute to the regulation of cholesterol efflux (Haidar, et al., 2001;Walter, et al., 1996). PC-PLC hydrolyzes PC producing diacylglycerol and phosphocholine and PC-PLD also hydrolyzes PC producing phosphatidic acid (PA) and choline. ApoA-I and a subspecies of HDL, HDL_3 are thought to activate these two phospholipases (Haidar, et al., 2001; Walter, et al., 1996) that in turn induces transport of cholesterol to the plasma membrane. In the presence of primary alcohols and PC-PLD a transphosphatidylation reaction occurs with production of a phosphatidylalcohol instead of PA. Cholesterol efflux has been reported to be inhibited by *n*-butanol in fibroblasts (Haidar, et al., 2001;Walter, et al., 1996). We found that ethanol increased activity of both PC-PLC and PC-PLD with the largest effect observed for PC-PLD. The increase in activities of PC-PLD and PC-PLC by ethanol are in agreement with earlier reports in studies of hepatocytes and fibroblasts (Kiss and Anderson, 1990;Pittner and Fain, 1992). An ethanol concentration of 25 mM had a greater effect on activity of both phospholipases as compared with 50 and 75 mM ethanol particularly with respect to PC-PLD. This concentration dependent effect of ethanol could be due to inhibition of upstream effectors of PC-PLD and PC-PLC such as protein kinase C, G-protein-coupled receptors, and apoA-I (Haidar, et al., 2001).

There is evidence that the Golgi complex is important in cholesterol efflux and that an isoform of PC-PLD may be associated with the Golgi complex (Heino, et al., 2000; Ktistakis, et al., 1995; Mendez, 1995; Siddhanata, et al., 2000). Ethanol may increase transport of cholesterol from the Golgi complex to the plasma membrane by stimulating activity of PC-PLD. It has been

shown in fibroblasts that 40 mM and 80 mM ethanol significantly reduced cholesterol content in the Golgi complex as compared to control cells (Igbavboa, et al., 2001). If cholesterol originating in the Golgi complex is transported to the plasma membrane, efflux of cholesterol from the plasma membrane may be hindered by ethanol-induced perturbation of apoA-I, HDL and cell surface proteins. However, it has been reported that 1-butanol (135 and 200 mM) inhibited release of nascent secretory vesicles of the Golgi complex that was attributed to a reduction of PA formation (Chen, et al., 1997; Siddhanata, et al., 2000). The Golgi complex structure was disassembled in the study using 200 mM butanol (Siddhanata, et al., 2000). 1-Butanol is more hydrophobic than ethanol, the membrane/buffer partition coefficient of 1-butanol is markedly higher as compared with ethanol (1.52 versus 0.096), and higher 1-butanol concentrations were used in that study compared with the ethanol study that could contribute to differences between effects of ethanol and butanol on the Golgi complex.

HDL and apoA-I induced cholesterol efflux in HASMC was inhibited by ethanol. Ethanol has also been shown to have similar effects on cholesterol efflux in rat fibroblasts (Avdulov, et al., 2000). Conversely, cholesterol efflux was increased in rat hepatoma cells incubated with plasma from human subjects who had consumed ethanol (30-40 g etoh/day/for 2-3 weeks) (Senault, et al., 2000; van der Gaag, et al., 2001). Alcohol amounts used in those studies were considered moderate. On the other hand , HDL isolated from alcoholic patients were less efficient in removing cholesterol from mouse macrophages (Rao, et al., 2000). Ethanol has both an acute effect and a chronic effect on cholesterol efflux. In the present study, effects of acute administration of ethanol at amounts observed in heavy drinkers and alcoholics inhibited cholesterol efflux. A potential consequence of elevated blood alcohol levels is that conversion of lipid-free apoA-I to mature HDL may be hindered. Moderate alcohol consumption stimulated

cholesterol efflux whereas HDL of alcoholic patients were less efficient in removing cholesterol from cells. Heavy drinkers and alcoholics may be at greater risk for vascular disease as a result of both the acute and chronic effects of ethanol on regulation of cholesterol in cells.

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FIGURE LEGENDS

Figure 1: Incorporation of DHE into HASMC. In all experiments, confluent cells 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 the Methods section. Data are means ± SEM (n = 3). (Inset) Confluent HASMC were incubated with different concentrations of DHE for 18 h. Cells were then harvested and processed, and DHE fluorescence was measured. Data are means ± SEM (n = 3).

Figure 2: HDL-mediated efflux of DHE from HASMC. Confluent cells were incubated with 3 μg of DHE/mL of cell culture medium for 18 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 DHE fluorescence was measured. Data are means ± SEM (n = 3). * $p \leq 0.02$ as compared with no HDL.

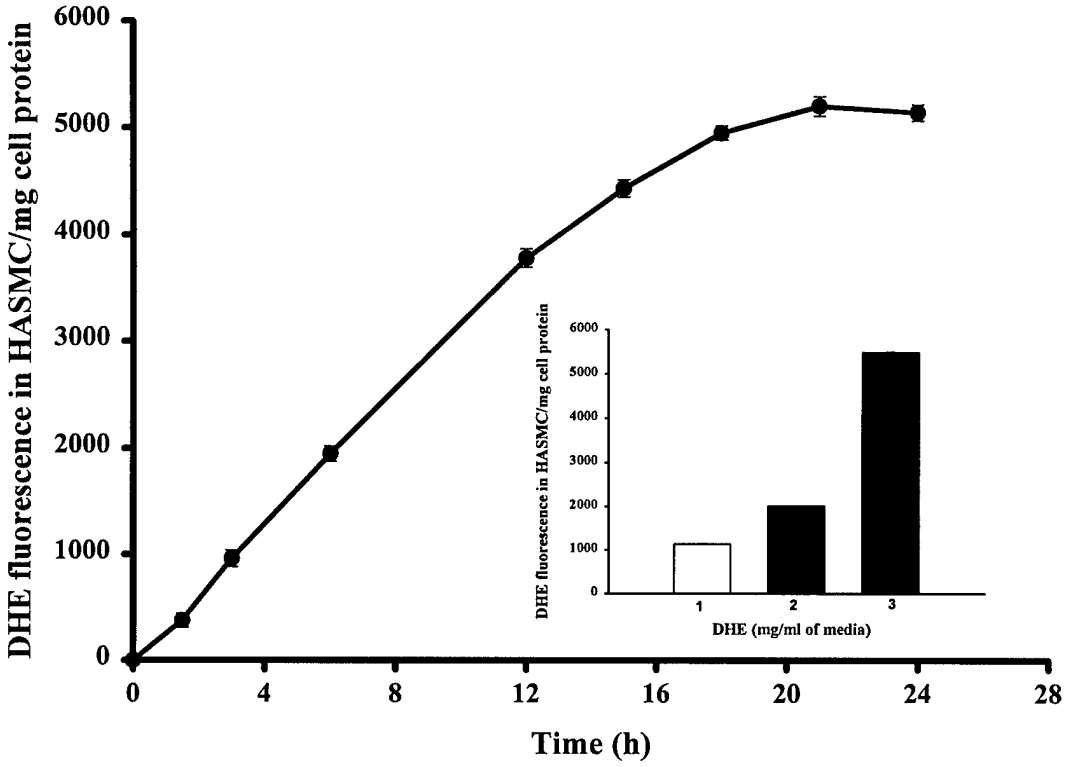
Figure 3: Ethanol Inhibits HDL-Mediated DHE Efflux from HASMC. Confluent HASMC were incubated with 3 μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25, 50, or 75 mM ethanol or no ethanol, after which 30 μg of human plasma 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 contained DHE but were not incubated with HDL or ethanol. Data are means ± SEM (n = 4). * $p \leq 0.04$ compared with HDL and no ethanol; ** indicates $p \leq 0.0001$ compared with control.

Figure 4: Ethanol Inhibits ApoA-I Mediated DHE Efflux from HASMC. Confluent HASMC were incubated with 3μg of DHE/mL of cell culture medium for 18 h. Cells were then washed twice using DHE-free medium and incubated for 30 min with 25, 50, 0r 75 mM ethanol or no

ethanol, after which 30 μg of human plasma apoA-I /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 contained DHE but were not incubated with apoA-I or ethanol. Data are means ± SEM (n = 3). $^{*}p \leq 0.001$ as compared to apoA-I no ethanol; $^{**}p \leq 0.0002$ as compared to control no apoA-I.

Figure 5: Effects of Ethanol on PC-PLD Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which cells were harvested as described in Methods section. 20 μg of cells were placed in 96 well plates and PC-PLD determined using the Amplex® red PC-specific PLD assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). $^{*}p \leq 0.004$ as compared to 0 ethanol; $^{**}p \leq 0.0001$ as compared to each condition.

Figure 6: Effects of Ethanol on PC-PLC Activity in HASMC. Confluent cells were incubated with different concentrations of ethanol for 2 h after which cells were harvested as described in Methods section. 20 μg of cells were placed in 96 well plates and PC-PLC determined using the Amplex® red PC-specific PLC assay kit. Samples were incubated for 60 min at 37°C. Fluorescence was measured with a microplate reader using an excitation wavelength of 542 nm and emission wavelength of 590 nm. Data are means ± SEM (n = 4). $^{*}p \leq 0.02$ as compared to each condition.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3000
2000
1000
0
0
4
8
12
16
20
24
28
Time (h)
DHE fluorescence in HASMC/mg cell protein
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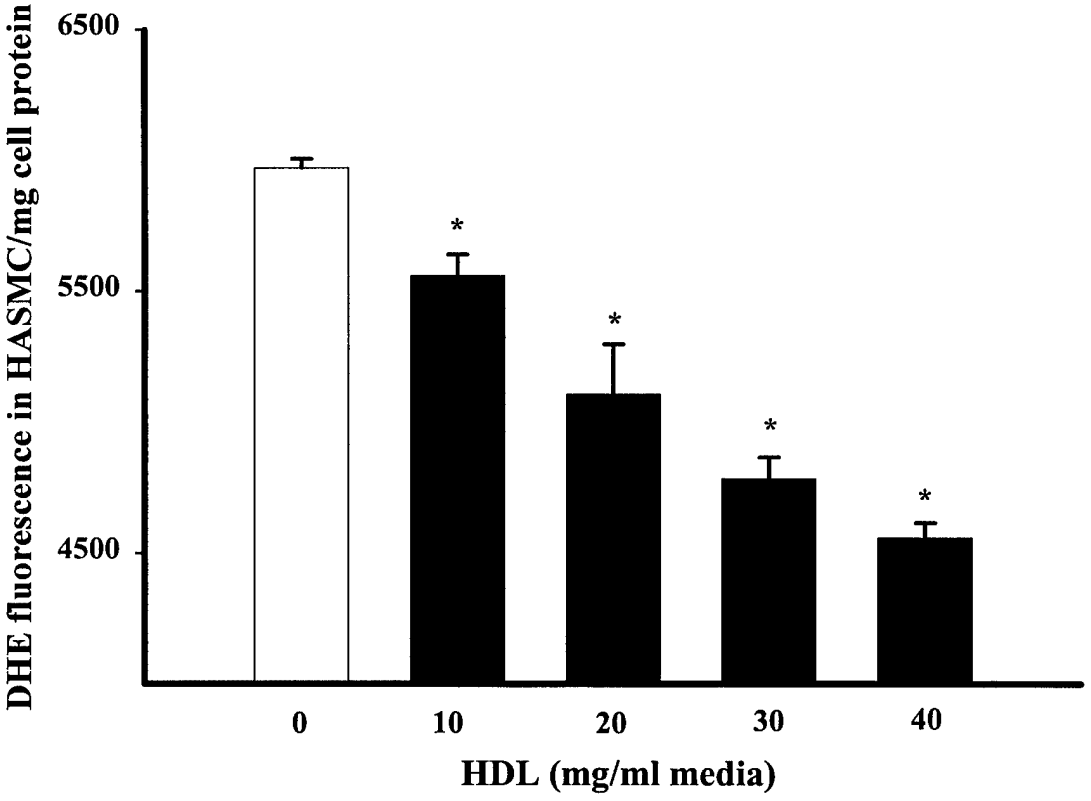
5000
4000
3000
2000
1000
0
1
2
3
DHE (mg/ml of media)


DHE fluorescence in HASMC/mg cell protein
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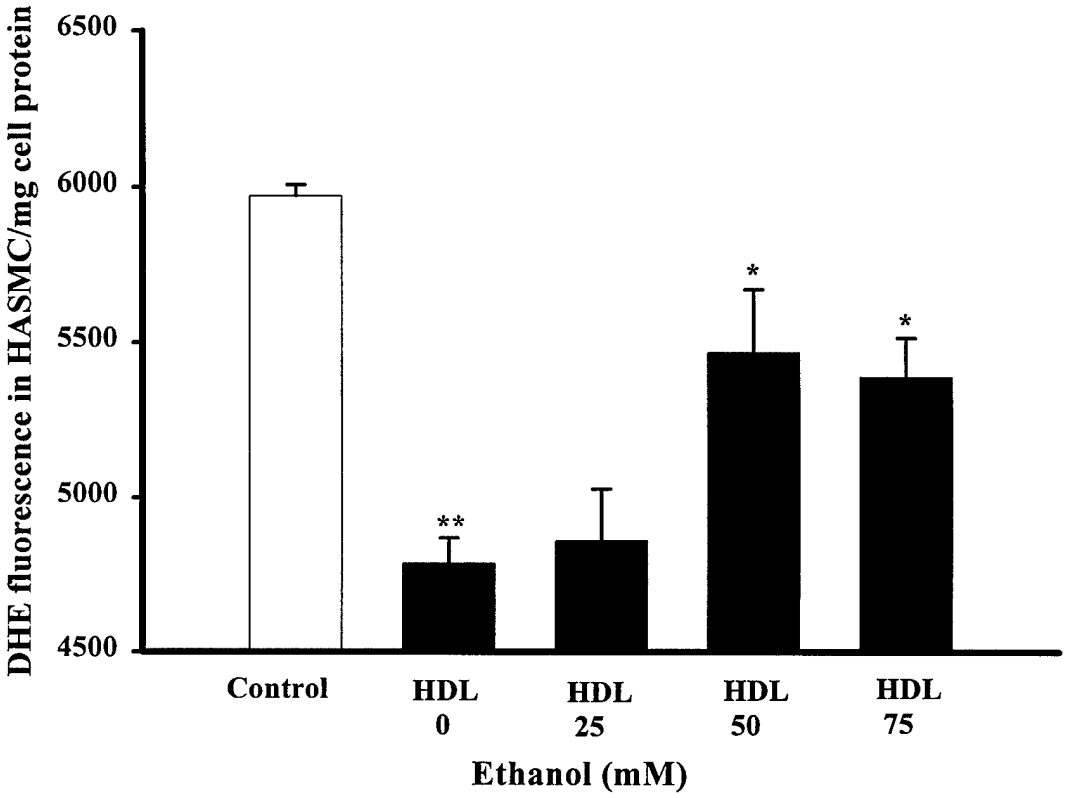
5500
4500
*
*
*
*
0
10
20
30
40
HDL (mg/ml media)


DHE fluorescence in HASMC/mg cell protein
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6000
5500
5000
4500
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*
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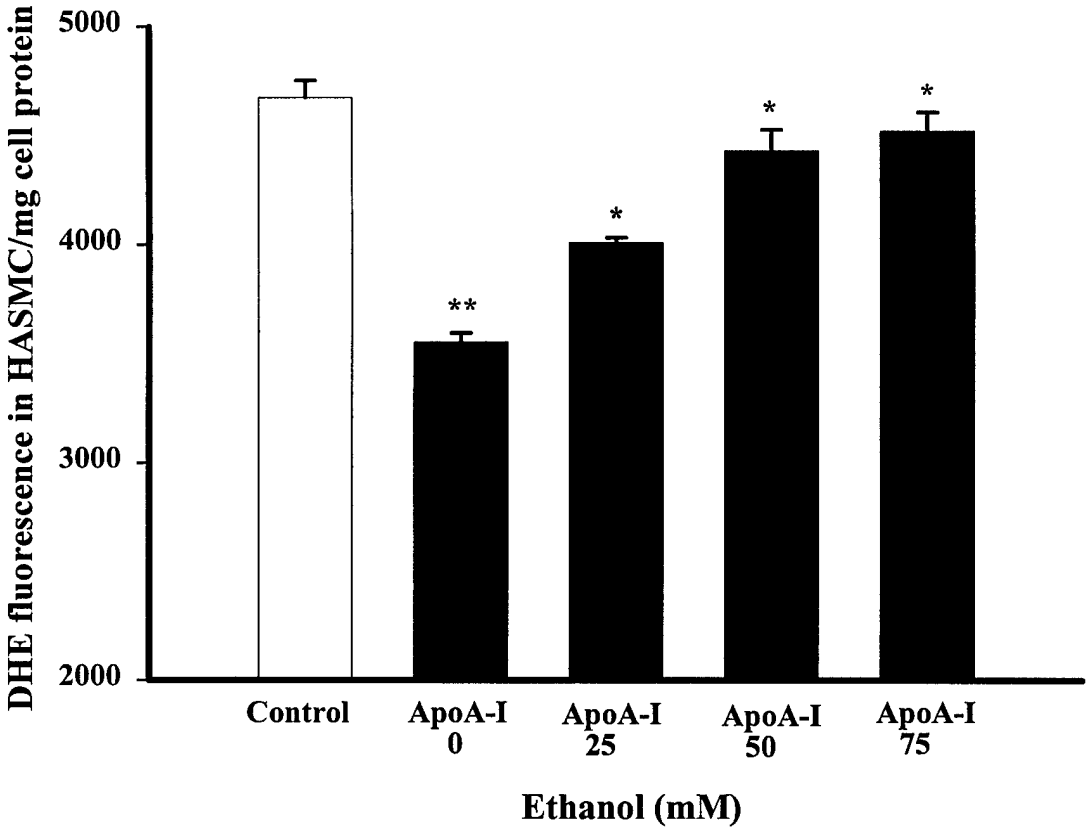
Control
HDL 0
HDL 25
HDL 50
HDL 75
Ethanol (mM)


DHE fluorescence in HASMC/mg cell protein
5000
4000
3000
2000
**
*
*
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Control
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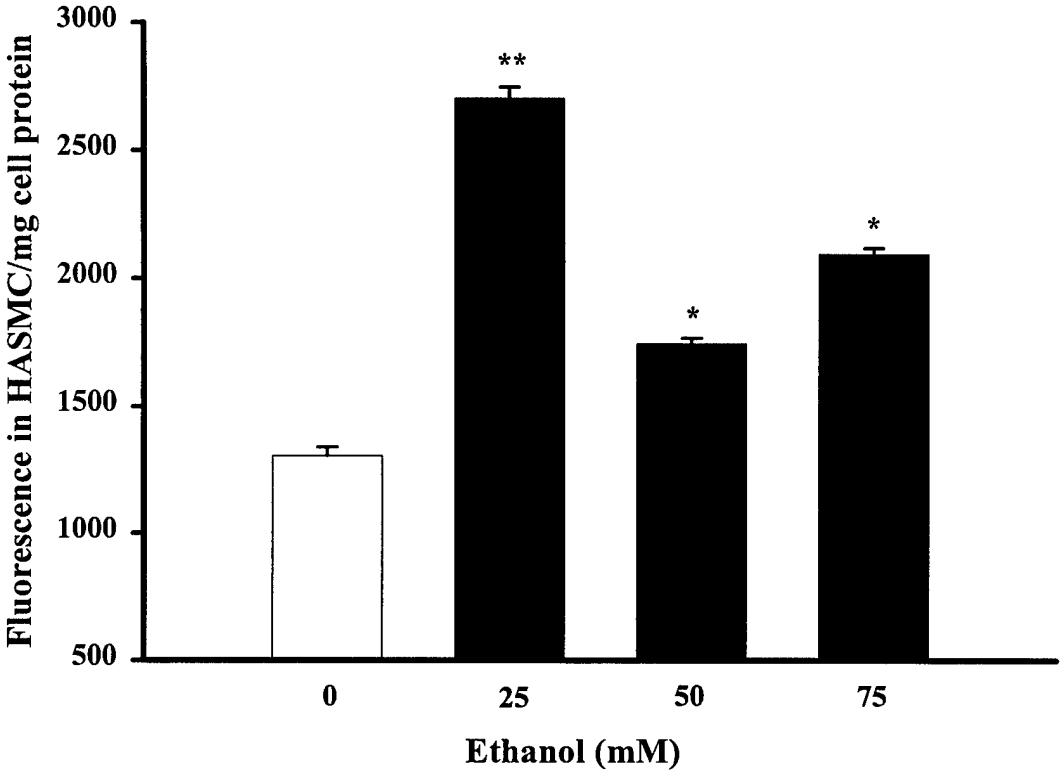
ApoA-I 25
ApoA-I 50
ApoA-I 75
Ethanol (mM)


Fluorescence in HASMC/mg cell protein
3000
2500
2000
1500
1000
500
**
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*
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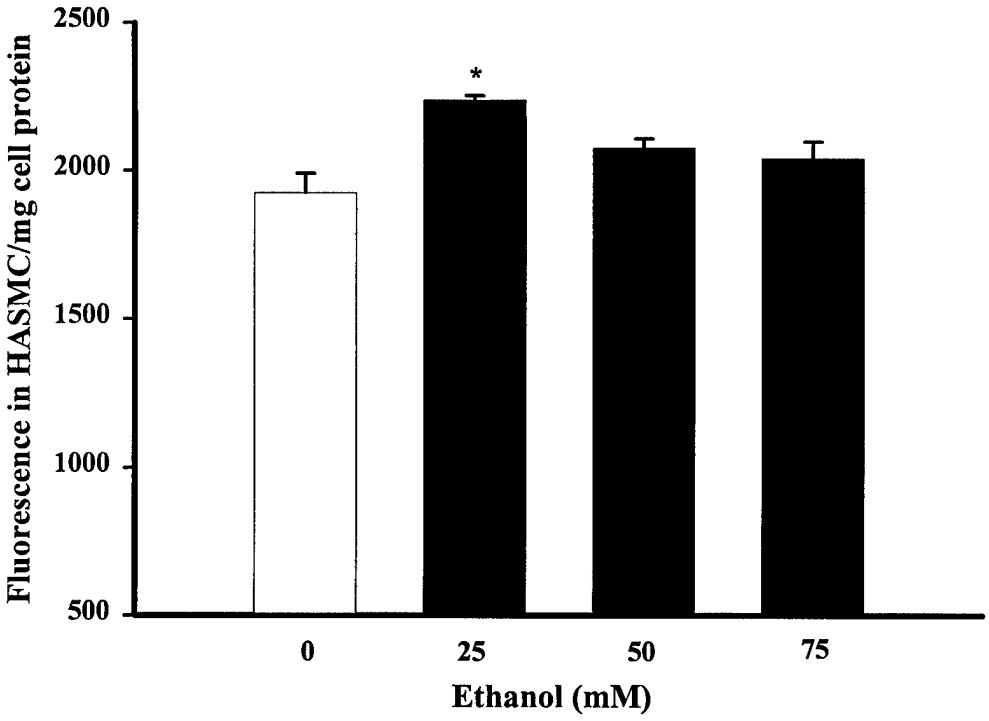
25
50
75
Ethanol (mM)


2500
2000
1500
1000
500
Fluorescence in HASMC/mg cell protein
*
0
25
50
75
Ethanol (mM)

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Cholesterol Distribution in the Golgi Complex of DITNC1 Astrocytes Is Differentially Altered by Fresh and Aged Amyloid β-Peptide-(1–42)*

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The Golgi complex plays an important role in cholesterol trafficking in cells, and amyloid β-peptides (Aβs) alter cholesterol trafficking. The hypothesis was tested that fresh and aged Aβ-(1–42) would differentially modify Golgi cholesterol content in DINTC1 astrocytes and that the effects of Aβ-(1–42) would be associated with the region of the Golgi complex. Two different methods were used to determine the effects of Aβ-(1–42) on Golgi complex cholesterol. Confocal microscopy showed that fresh Aβ-(1–42) significantly increased cholesterol and that aged Aβ-(1–42) significantly reduced cholesterol content in the Golgi complex. Isolation of the Golgi complex into two fractions using density gradient centrifugation showed effects of aged Aβ-(1–42) similar to those observed with confocal microscopy but revealed the novel finding that fresh Aβ-(1–42) had opposite effects on the two Golgi fractions suggesting a specificity of Aβ-(1–42) perturbation of the Golgi complex. Phosphatidylcholine-phospholipase D activity, cell membrane cholesterol, and apolipoprotein E levels were associated with effects of fresh Aβ-(1–42) on cholesterol distribution but not with effects of aged Aβ-(1–42), arguing against a common mechanism. Extracellular Aβ-(1–42) targets the Golgi complex and disrupts cell cholesterol homeostasis, and this action of Aβ-(1–42) could alter cell functions requiring optimal levels of cholesterol.

Several different lines of evidence point to a potentially important but not well understood interaction between Alzheimer's disease (AD)[1] and cholesterol (for reviews, see Refs. 1–4). Apolipoprotein E4, a protein that binds and transports cholesterol and other lipids, has been identified as a risk factor for familial and sporadic AD (5, 6). Patients on specific inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase have a lower risk of developing AD as compared with individuals not taking those drugs (7, 8). A metabolite of brain cholesterol, (24*S*)-hydroxycholesterol, was elevated in cerebral spinal fluid of AD patients compared with control subjects (9).

Amyloid β-peptide (Aβ), the main component of neuritic plaques seen in brains of AD patients, interacts with cholesterol. This interaction is reciprocal. Cholesterol levels modulate amyloid precursor protein and Aβ synthesis (10–13). Conversely Aβ alters cholesterol dynamics. Cholesterol modulates the actions of Aβ on brain membrane fluidity (14–16). Aβ species (25–35, 1–40, and 1–42) increased the internalization of apolipoprotein E (apoE) complexed with cholesterol into neurons (17). Aβ-(1–42) increased apoE levels in astrocytes (18). Cholesterol efflux from rat hippocampal neurons to cyclodextrin was enhanced by Aβ-(1–40), and these results were attributed to redistribution of cholesterol to the plasma membrane (19). The Golgi complex plays an important role in cholesterol trafficking (20, 21), and Aβ could act on cholesterol homeostasis in the Golgi complex. Chemical inhibitors known to act on the Golgi complex alter cholesterol trafficking (20). Furthermore it has been shown that phosphatidylcholine-phospholipase D (PC-PLD) contributes to regulation of cholesterol efflux and that PC-PLD is associated with the Golgi complex (20–24). The Aβ peptide fragment 25–35 increased PLD activity in LA-N-2 cells (25). The effects of Aβ on cholesterol in the Golgi complex may also be dependent on whether Aβ is fresh or aged. Recently it was reported that oligomeric Aβ-(1–40), but not monomeric Aβ-(1–40), stimulated release of cholesterol, phospholipids, and GM1 ganglioside from neurons (26). Aged Aβ-(1–40), but not fresh Aβ-(1–40), preferentially binds cholesterol as compared with fatty acids or phosphatidylcholine (27) that might enhance removal of lipids from cell compartments. Fresh Aβ-(1–40) was reported to be located in the hydrophobic area of synaptic plasma membrane, whereas Aβ-(1–40) aged for 48 h was intercalated adjacent to the phospholipid polar head group region (28).

The current study tested the hypotheses that Aβ-(1–42) modifies Golgi complex cholesterol homeostasis in astrocytes and that the effects of Aβ-(1–42) were dependent on whether Aβ-(1–42) was fresh or aged. Gel electrophoresis of fresh Aβ-(1–42) and Aβ-(1–42) aged for 48 h showed that aged Aβ-(1–42) consisted of ~74% of the protein as a tetramer, whereas the fresh Aβ-(1–42) showed only 11% of the protein as a tetramer (28), and such structural differences could alter the behavior of Aβ. The effects of Aβ-(1–42) on Golgi complex cholesterol could involve activity of PC-PLD, and this possible action of Aβ-(1–

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[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, amyloid β-peptide; apo, apolipoprotein; PC, phosphatidylcholine; PLD, phospholipase D; NBD-cholesterol, 22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino-23,24-bisnor-5-cholen-3-β-ol); PBS, phosphate-buffered saline; BODIPY TR ceramide, *N*-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-yl)phenoxy)acetyl)sphingosine; GM1, II^{3}NeuAc-GgOse$_4$Cer (where GgOse$_4$Cer is gangliotetraosyl ceramide); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

42) was examined. In addition, it has been reported that apoE levels were increased in astrocytes treated with aged Aβ-(1–42) (18), and such effects might alter cholesterol distribution in cells. ApoE levels also were determined. Experiments were conducted using fresh and aged Aβ-(1–42) in immortalized DITNC1 astrocytes (29). Two different methods were used to determine the effects of Aβ-(1–42) on Golgi cholesterol content of astrocytes: 1) confocal microscopy using the fluorescent cholesterol analogue NBD-cholesterol and a fluorescent marker for the Golgi complex, BODIPY TR ceramide (30); and 2) isolation of the Golgi complex into two different fractions thought to represent different regions of the Golgi complex using sucrose density gradient centrifugation (31, 32). The results presented herein provide new insights into interaction of Aβ and cholesterol. We show that fresh Aβ-(1–42) has a strikingly different effect on cholesterol levels in the Golgi complex of astrocytes as compared with aged Aβ-(1–42). Incubation of astrocytes with fresh Aβ-(1–42) significantly increased cholesterol levels, whereas aged Aβ-(1–42) significantly reduced cholesterol levels in the Golgi complex as shown by fluorescent probes and confocal microscopy. The reverse peptide Aβ-(42–1), either fresh or aged, did not alter Golgi complex cholesterol levels, which argues against a nonspecific action of Aβ-(1–42). Isolation of the astrocyte Golgi complex into different fractions revealed the novel finding that the effects of fresh Aβ-(1–42) on cholesterol content were opposite on the two Golgi fractions, whereas aged Aβ-(1–42) had effects similar to those observed with confocal microscopy. PC-PLD activity, cell membrane cholesterol, and apoE levels of astrocytes were associated with effects of fresh Aβ-(1–42) on cholesterol levels but not with effects of aged Aβ-(1–42), suggesting different mechanisms for actions of fresh and aged Aβ-(1–42) on Golgi complex cholesterol. Our data suggest that extracellular Aβ-(1–42) specifically targets the Golgi complex and disrupts cholesterol homeostasis.

EXPERIMENTAL PROCEDURES

Materials—NBD-cholesterol (22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-β-ol), BODIPY TR ceramide, and the Amplex® Red phosphatidylcholine-specific phospholipase D (A-12219) assay kit were obtained from Molecular Probes (Eugene, OR). Aβ-(1–42) and Aβ-(42–1) were purchased from Bachem California Inc. (Torrance, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). UDP-[6-^{3}H]galactose was purchased from Amersham Biosciences. The Aβ-(1–42) enzyme-linked immunosorbent assay kit (catalogue no. 17711) was obtained from Immuno-Biological Laboratories (Minneapolis, MN). All other chemicals unless specifically mentioned were purchased from Sigma.

Cell Culture—DITNC1 rat astrocytes were purchased from American Type Culture Collection (Manassas, VA). These cells have been shown to have the phenotypic characteristics of type 1 astrocytes (29). Astrocyte cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% glutamate for 2 days until confluence and maintained at 37 °C in 5% CO_2 and at 90% relative humidity. The cells at 80–85% confluence were treated with medium containing 1% lipoprotein-deficient serum replacing the 10% fetal bovine serum and incubated for 16 h prior to experimentation. All experiments were done using confluent astrocytes.

Incubation of Cells with Fluorescent Labeled Probes and Aβ-(1–42)—Astrocytes were grown on Lab-Tek™ chamber slides, incubated with NBD-cholesterol (8 μM) for 1 h, and then washed three times with 1 ml of phosphate-buffered saline (PBS). Fresh Aβ-(1–42) (1 μM) or Aβ-(1–42) (1 μM) preincubated for 48 h (aged) was added to cells and incubated for different time periods (30, 60, and 120 min). We have reported previously that incubation of Aβ-(1–42) for 48 h resulted in ~74% of the peptide in a tetrameric form (28). In some experiments the reverse peptide Aβ-(42–1) (fresh or aged for 48 h) was incubated with cells. Following incubation with Aβ, cells were rinsed three times with PBS, and the Golgi marker BODIPY TR ceramide (2 μM) was added and incubated with the cells for 1 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde, and mounted for confocal microscopy using Gel/Mount (Biomeda Corp., Foster City, CA).

Laser Scanning Confocal Microscopy—Confocal fluorescence imaging was performed on an Olympus Fluoview laser scanning confocal imaging system (Olympus America Inc., New York). Images were captured using multiple photomultiplier tubes regulated by Fluoview 2.0 software (Olympus). Excitation of the fluorescent probes was accomplished using 15-milliwatt krypton-argon lasers with 5-milliwatt output. An Olympus BX 50 fluorescent microscope was used to capture the images using an oil immersion objective. NBD-cholesterol was excited at 488 nm, and emission was recorded at 540 nm. BODIPY TR ceramide was excited at 568 nm, and emission was recorded at 598 nm. The captured images for the red and green channels were merged and appeared yellow, which is indicative of colocalization. Red and green are additive, generating yellow to orange in RGB (red-green-blue) color space (33). Quantitative analysis of the colocalization of NBD-cholesterol and BODIPY TR ceramide was determined by image processing using MetaMorph imaging system V4.3 from Universal Imaging Corp. (Downingtown, PA) as described previously (33, 34) and expressed as percent colocalization.

Isolation of Golgi Fractions—Isolation of the Golgi complex was accomplished using sucrose density gradient centrifugation (32). Confluent astrocytes were treated with fresh and aged Aβ-(1–42) as described above. Cells were harvested and washed twice with PBS. The cells were suspended in G buffer (10 mM Tris-HCl, 0.25 M sucrose, 2 mM $MgCl_2$, pH 7.4) containing 10 mM $CaCl_2$. Leupeptin and phenylmethylsulfonyl fluoride were added to the cell suspension to inhibit proteolytic enzymes prior to homogenization. The cells were homogenized in a Potter-type homogenizer with 20–30 strokes using a serrated homogenizing pestle. The homogenate was centrifuged at 5,000 × *g* for 10 min. The pellet was suspended and homogenized in 1.4 M sucrose and overlayered with 0.8, 1.0, and 1.2 M sucrose gradients in ultracentrifuge tubes. Samples were then centrifuged at 95,000 × *g* for 2.5 h in a SW28 rotor in a Beckman L8–70 ultracentrifuge. Each interface was carefully removed, diluted with G buffer, and centrifuged in an SS34 rotor at 45,900 × *g* for 20 min. Each pellet was suspended in buffer and used for the experiments. Protein concentrations of the fractions were determined using the Bradford assay. Cholesterol content in each fraction was determined enzymatically in a microassay using a diagnostic kit from Roche Molecular Biochemicals (35) and procedures reported by our laboratory (36–38).

Galactosyltransferase Activity—A marker enzyme of the Golgi complex is galactosyltransferase, and activity of this enzyme was measured using procedures reported previously (32, 39). The incubation mixture contained 7 mg/ml ovalbumin, 2 mM ATP, 200 mM $MgCl_2$, 0.2% Triton X-100, and 50 mM Tris-HCl (pH 6.8). The reaction was initiated by adding [^{3}H]UDP-galactose to samples and incubated for 30 min, and the reaction was stopped by adding ice-cold 24% trichloroacetic acid. The precipitate was pelleted at 20,800 × *g* and washed three times with 12% trichloroacetic acid. Samples were solubilized in 5% SDS, and disintegrations/min were counted using a scintillation counter.

PC-PLD Activity—PC-PLD activity of control astrocytes and astrocytes that had been incubated with either fresh or aged Aβ-(1–42) for different time periods (30, 60, 90, and 120 min) was quantified using the Amplex Red PC-PLD assay kit (Molecular Probes). The intensity of the Amplex Red complex formed was measured in a fluorescence microplate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 542 nm and an emission wavelength of 590 nm.

Measurement of Aβ-(1–42)—Quantitative determination of Aβ-(1–42) in the G1 and G2 fractions was carried out using an enzyme-linked immunosorbent assay kit from Immuno-Biological Laboratories. This kit uses a solid phase sandwich enzyme-linked immunosorbent assay protocol with anti-human Aβ-(38–42) rabbit IgG as primary antibody in a precoated plate and horseradish peroxidase-conjugated anti-human Aβ-(11–28) mouse IgG as secondary conjugated antibody. Briefly, standards and test samples were diluted in a buffer consisting of 1% bovine serum albumin and 0.05% Tween 20 in PBS. Test samples as well as sample and reagent blanks were added to the precoated plate and incubated overnight at 4 °C. After binding, the wells were washed (seven times) prior to adding conjugated secondary antibody and incubated for 1 h at 4 °C. After washing (nine times), the chromogen containing tetramethylbenzidine was added and incubated for 30 min at room temperature. Color reaction was terminated by the stop solution, and measurement was performed at 450 nm using a Packard Fusion AlphaScreen microplate analyzer (Packard Instrument Co.). In each assay, a standard curve was constructed after subtracting absorbance of the test sample blank. Data for test samples were converted to ng of Aβ-(1–42)/mg of protein.

ApoE Isolation—Media of control cells and cells treated with either

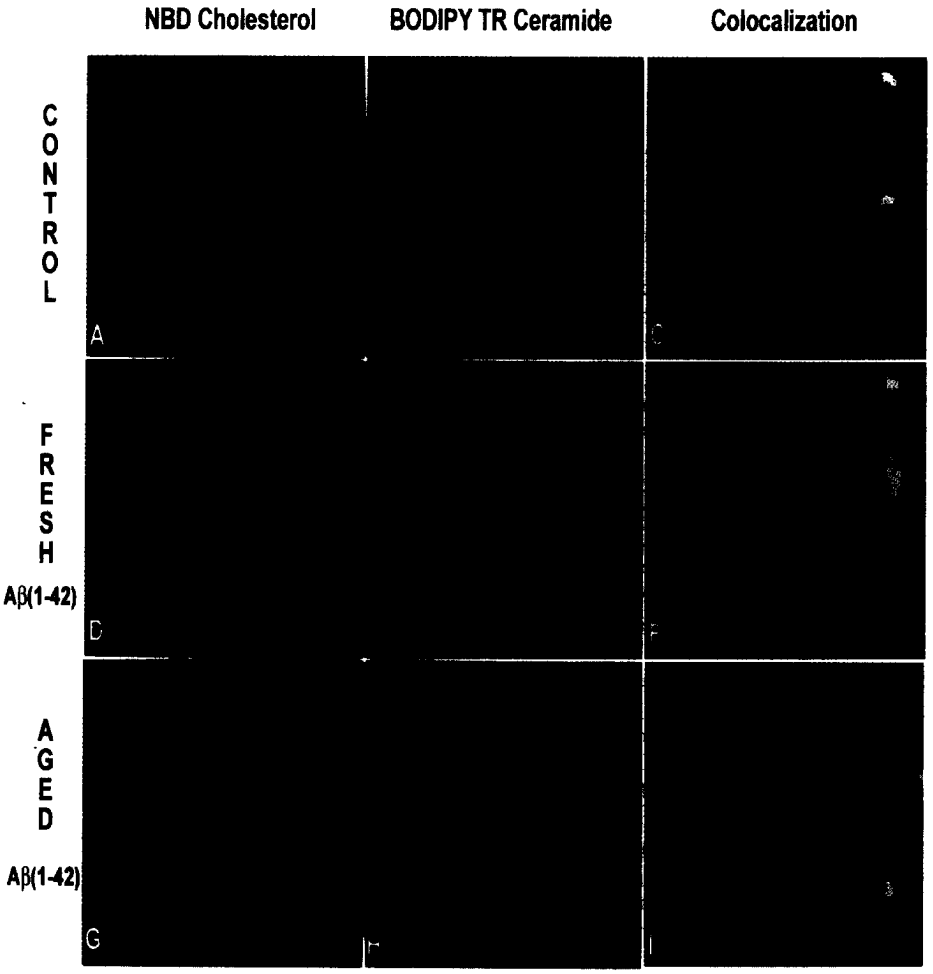

FIG. 1. **Localization of cholesterol in the Golgi complex of astrocytes incubated with fresh and aged Aβ-(1–42) for 120 min.** Shown are confocal images of control astrocytes labeled with NBD-cholesterol (*panel A, green*) and BODIPY TR ceramide (*panel B, red*). Colocalization is shown in *panel C*. Shown are confocal images of astrocytes treated with fresh Aβ-(1–42), NBD-cholesterol (*panel D, green*), and BODIPY TR ceramide (*panel E, red*). Colocalization is shown in *panel F*. Shown are confocal images of astrocytes treated with aged Aβ-(1–42), NBD-cholesterol (*panel G, green*), and BODIPY TR ceramide (*panel H, red*). Colocalization is shown in *panel I*.

fresh or aged Aβ-(1–42) were removed, and phenylmethylsulfonyl fluoride was added as a protease inhibitor. The media were centrifuged at 800 × *g* for 5 min, and the resulting supernatant was treated with 15% trichloric acid to precipitate protein, washed with cold methanol, dried, and kept at −20 °C for later use. Cells were harvested with trypsin/EDTA, and protein values were determined using the Bradford method with ovalbumin standards. Cells were treated with lysing buffer containing 8 M urea, 5% SDS, and 5% β-mercaptoethanol. For Western blot analysis of lysates and conditioned media, 1 μl of 1 M dithiothreitol and 1 μl of 0.2% bromphenol solution was added to every 50 μl of lysing buffer before use, and samples were kept at 70 °C for 30 min. 100–200 μg of protein were electrophoresed on a 7.5% SDS, Tricine, HCl precast gel (Bio-Rad), and proteins were transferred to a nitrocellulose membrane and incubated with monoclonal mouse anti-rat apoE (1:250) from BD Laboratory (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000) was used as a secondary antibody (Pierce). Immunoreactivity was visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce). Band density was quantitated by densitometry using the Eagle Eye II video system and EagleSight software (Stratagene, La Jolla, CA).

RESULTS

Aβ-(1–42) Modifies Cholesterol Distribution in the Golgi Complex as Revealed by Confocal Microscopy—Experiments in this study tested the hypothesis that Aβ-(1–42) would modify cholesterol distribution in the Golgi complex of astrocytes and that the effects of Aβ-(1–42) on cholesterol distribution would be dependent on whether Aβ-(1–42) was fresh or aged. Two different methods were used to determine cholesterol distribution in the Golgi complex. 1) A fluorescent cholesterol analogue, NBD-cholesterol, and a fluorescent marker for the Golgi complex, BODIPY TR ceramide, were used and imaged with confocal microscopy; and 2) sucrose density gradient centrifugation was used to isolate two different fractions of the Golgi complex, and cholesterol levels were determined enzymatically.

BODIPY TR ceramide, NBD-cholesterol, and confocal microscopy were used to initially examine the effects of fresh and aged Aβ-(1–42) on Golgi cholesterol content of astrocytes. Cells were incubated with Aβ-(1–42) for different time periods (30, 60, and 120 min). Cell viability as measured by trypan blue was not altered by incubation of cells with fresh or aged Aβ-(1–42) for 120 min. Means ± S.E. of the percentage of viable cells for control astrocytes, astrocytes incubated with fresh Aβ-(1–42), and astrocytes incubated with aged Aβ-(1–42) were 96.83 ± 0.58, 94.2 ± 1.5, and 97.83 ± 0.6, respectively. Confocal images of colocalization of NBD-cholesterol and BODIPY TR ceramide in control astrocytes and astrocytes incubated with fresh and aged Aβ-(1–42) for 120 min are shown in Fig. 1. *Panel C* shows colocalization of the two fluorescent probes in control astrocytes. It can be seen in *panel F* that colocalization was greater in astrocytes treated with fresh Aβ-(1–42), whereas aged Aβ-(1–42) (*panel I*) reduced colocalization as compared with control astrocytes and astrocytes incubated with fresh Aβ-(1–42). Analysis of colocalization data using MetaMorph software and expressed as percent colocalization shows that fresh Aβ-(1–42) ($p \leq 0.001$) increased colocalization in the Golgi complex of astrocytes as compared with control astrocytes (38 *versus* 23%) at the 120-min incubation period (Fig. 2). The percent colocalization of the fluorescent probes was significantly ($p \leq 0.01$) less when astrocytes were incubated with aged Aβ-(1–42) (15 *versus* 23%) for 120 min as compared with control astrocytes (Fig. 2). Shorter incubation times with Aβ-(1–42) did not significantly alter colocalization, although it can be seen in Fig. 2

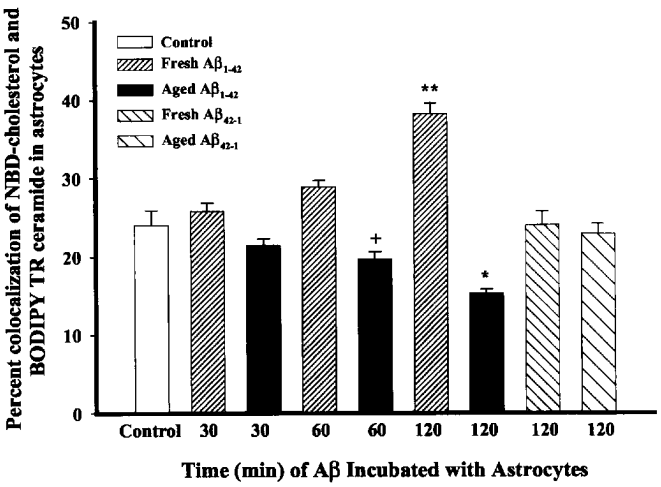

FIG. 2. **Percent colocalization of BODIPY TR ceramide and NBD-cholesterol in astrocytes incubated with fresh and aged Aβ-(1–42) for different time periods and astrocytes incubated with Aβ-(42–1) for 120 min.** Quantitative measurements of colocalization of NBD-cholesterol and BODIPY TR ceramide were determined using MetaMorph software and expressed as percent colocalization. Data are means ± S.E. (n = 3 separate experiments). *, $p \leq 0.01$; **, $p \leq 0.001$ as compared with control astrocytes.

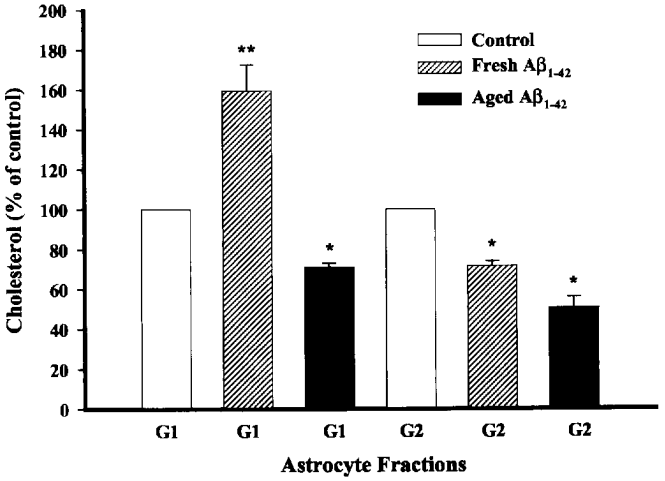

FIG. 3. **Changes in the distribution of cholesterol in the Golgi complex of astrocytes incubated with fresh and aged Aβ-(1–42).** Astrocytes were incubated with Aβ-(1–42) for 120 min after which time cells were harvested, and the Golgi complex was isolated using sucrose density centrifugation as described under "Experimental Procedures." The G1 fraction is the band at the 0.8–1.0 M sucrose gradient, and the G2 fraction is the band at the 1.0–1.2 M sucrose gradient. Cholesterol was determined enzymatically, and data are expressed as percentage of control. Values are means ± S.E. (n = 3). *, $p \leq 0.001$; **, $p \leq 0.0001$ as compared with control fractions.

that fresh Aβ-(1–42) was increasing colocalization and aged Aβ-(1–42) was reducing colocalization at the shorter incubation times. Colocalization was not altered by a lower concentration (1 nmol) of fresh Aβ-(1–42) or aged Aβ-(1–42). Means ± S.E. of percent colocalization of NBD-cholesterol and BODIPY TR ceramide were 23.71 ± 3.7, 25.82 ± 4.7, and 23.33 ± 2.1 for control astrocytes, astrocytes incubated with fresh Aβ-(1–42), and astrocytes incubated with aged Aβ-(1–42), respectively. To determine whether the effects of Aβ-(1–42) were specific and not attributable to a nonspecific perturbation of the Golgi complex we incubated astrocytes for 120 min with the reverse peptide Aβ-(42–1) that was either fresh or aged for 48 h. Neither fresh nor aged Aβ-(42–1) had an effect on the percent colocalization of BODIPY TR ceramide and NBD-cholesterol when compared with control astrocytes or with each other (Fig. 2).

UDP-galactosyltransferase Activity and Fluorescence of BODIPY TR Ceramide in Golgi Complex Fractions—Confocal microscopy revealed that Aβ-(1–42) altered the colocalization of NBD-cholesterol and BODIPY TR ceramide, a marker for the Golgi complex, and these data were interpreted as Aβ-(1–42) altering Golgi cholesterol levels. The Golgi complex is heterogenous in structure and function and may not be equally affected by Aβ-(1–42). To further define the effects of fresh and aged Aβ-(1–42) on cholesterol distribution in the Golgi complex of astrocytes, the Golgi complex was isolated using sucrose density centrifugation, and two different fractions were obtained that are thought to represent different regions of the Golgi complex (32): 1) the light Golgi fraction (G1), which is the band at the 0.8–1.0 sucrose gradient interface; and 2) the Golgi fraction 2 (G2), which is the band at the 1.0–1.2 sucrose gradient interface. Enrichment of UDP-galactosyltransferase activity and fluorescence of BODIPY TR ceramide have been used previously as markers for the Golgi complex (30, 32, 40, 41), and these markers were used in the present study. The means ± S.E. of UDP-galactosyltransferase activity (nmol/30 min/mg of protein) in astrocyte homogenate, G1 fraction, and G2 fraction were 0.019 ± 0.004, 2.751 ± 0.086, and 1.972 ± 0.129, respectively. UDP-galactosyltransferase activity was significantly higher ($p < 0.01$) in the G1 fraction as compared with the G2 fraction and was several orders of magnitude higher in both the G1 and G2 fractions in contrast to the homogenate. Enrichment of UDP-galactosyltransferase activity in the G1 fraction as compared with the G2 fraction has been reported previously (32). BODIPY TR ceramide, a fluorescent Golgi complex marker, had a similar distribution in the G1 and G2 astrocyte fractions as was observed for UDP-galactosyltransferase activity (data not shown).

Aβ-(1–42) Modifies Cholesterol Distribution in the Golgi Complex Fractions as Revealed by Sucrose Density Centrifugation—We next examined the effects of Aβ-(1–42) on cholesterol content of the G1 and G2 fractions. The effects of fresh and aged Aβ-(1–42) on cholesterol in the G1 fraction were similar to effects we observed using confocal microscopy. Fresh Aβ-(1–42) significantly ($p < 0.0001$) increased cholesterol, whereas aged Aβ-(1–42) significantly ($p < 0.001$) reduced cholesterol in the G1 fraction (Fig. 3). The magnitude of effects of fresh Aβ-(1–42) on cholesterol in the G1 fraction was greater than that of aged Aβ-(1–42), and these results were similar to those observed with confocal microscopy.

Fig. 3 shows that fresh Aβ-(1–42) had an opposite effect on cholesterol in the G2 fraction as compared with the G1 fraction. There was a significant ($p < 0.001$) reduction in cholesterol in the G2 fraction when incubated with fresh Aβ-(1–42). On the other hand, aged Aβ-(1–42) had a similar effect on cholesterol in the G2 fraction as observed in the G1 fraction, resulting in a significant ($p < 0.001$) reduction in cholesterol (Fig. 3). Differences in effects of fresh and aged Aβ-(1–42) on cholesterol levels in the Golgi complex could be attributed to differences in amounts of fresh and aged Aβ-(1–42) that reach the Golgi complex. Levels of fresh Aβ-(1–42) were higher in the G1 and G2 fractions (0.21 ± 0.03 and 0.22 ± 0.1 ng/mg of protein, respectively) as compared with aged Aβ-(1–42) in the G1 and G2 fractions (0.11 ± 0.05 and 0.16 ± 0.04 ng/mg of protein, respectively). These differences in Aβ-(1–42) levels, however, were not statistically significant.

Both colocalization data and data of the G1 fraction showed that fresh Aβ-(1–42) increased cholesterol and aged Aβ-(1–42) reduced cholesterol in the Golgi complex of astrocytes. The cell membrane is enriched in cholesterol and could be a source of cholesterol in cells treated with fresh Aβ-(1–42). Table I shows

TABLE I
Total cholesterol, free cholesterol, and the molar ratio of free to total cholesterol in cell membranes of astrocytes incubated with fresh and aged Aβ-(1–42)

Treatment	Total cholesterol	Free cholesterol	Free/total
	μmol/mg protein	*μmol/mg protein*	
Control	9.59 ± 0.13	8.26 ± 0.65	0.87 ± 0.07
Fresh Aβ-(1–42)	6.37 ± 0.19[a]	5.84 ± 0.36[b]	0.92 ± 0.02
Aged Aβ-(1–42)	7.97 ± 0.24[b,c]	7.32 ± 0.26[c]	0.92 ± 0.06

[a] $p \leq 0.01$ as compared with control.
[b] $p \leq 0.05$ as compared with control.
[c] $p \leq 0.05$ as compared with fresh Aβ-(1–42), $n = 3$.

that fresh Aβ-(1–42) significantly reduced both total and free cholesterol in the cell membrane as compared with control astrocytes. Total cholesterol was reduced by 34% and free cholesterol by 30% when treated with fresh Aβ-(1–42). Aged Aβ-(1–42) also significantly reduced total and free cholesterol (17 and 11%, respectively) in the cell membrane (Table I), but effects were much less than those observed for fresh Aβ-(1–42). The ratio of free cholesterol to total cholesterol was used as an estimate of esterified cholesterol. Data in Table I show that the ratios were similar among the three groups and did not differ significantly. It also was noted that cholesterol content of the astrocyte homogenate fraction did not differ among the three groups.

Aβ-(1–42) Inhibits PC-PLD Activity—PC-PLD has been shown to be involved in regulating cholesterol efflux from cells (42) and in release of secretory vesicles from the Golgi complex (23, 43), and an isoform of PC-PLD has been reported to be associated with the Golgi complex (20, 22). To begin to address a potential mechanism whereby Aβ-(1–42) alters cholesterol content in the Golgi complex, we examined the effects of fresh and aged Aβ-(1–42) on activity of PC-PLD in astrocytes. Fig. 4 (*panels A* and *B*) reveals that fresh and aged Aβ-(1–42) significantly ($p < 0.001$) inhibited PC-PLD activity at each incubation time period. There was a small diminution of effects of Aβ-(1–42) on PC-PLD activity with increasing incubation time. PC-PLD activity was significantly ($p < 0.001$) higher after 90 and 120 min of incubation *versus* 30 min of incubation for fresh Aβ-(1–42) and after 120 min for aged Aβ-(1–42) (Fig. 4, *panels A* and *B*). The effects of fresh Aβ-(1–42) on PC-PLD activity were consistent with the increase in cholesterol in the Golgi complex, but PC-PLD activity does not explain the effects of aged Aβ-(1–42) on Golgi complex cholesterol.

Effects of Fresh and Aged Aβ-(1–42) on ApoE Levels in Astrocytes—It has been reported previously that Aβ-(1–42) increased astrocyte activation, increased levels of apoE and apoJ in the cell, and reduced levels of the two apolipoproteins in the medium (18). The effects of activation were proposed to primarily involve apoE and its receptors. Changes in Aβ-(1–42)-induced levels of apoE and release of apoE could have an effect on cholesterol content by sequestering cholesterol in the cell. Fig. 5 (*inset*) shows that apoE under reducing conditions can be seen as approximately a 34-kDa monomer, and this finding is consistent with apoE expression in astrocytes (18). Fresh Aβ-(1–42) significantly ($p \leq 0.01$) increased apoE levels by 32% in astrocytes as compared with control cells (Fig. 5). Aged Aβ-(1–42) appeared to reduce apoE levels, but this difference was not statistically significant when compared with control cells. ApoE was not detected in the conditioned media from control and Aβ-(1–42)-treated cells (data not shown).

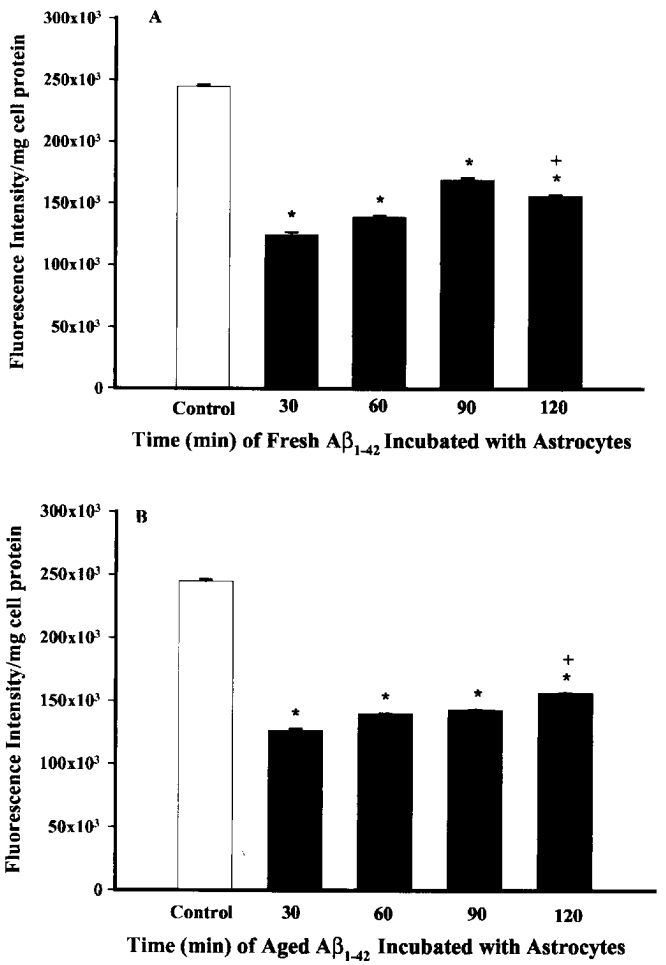

FIG. 4. **Fresh and aged Aβ-(1–42) inhibit PC-PLD activity in astrocytes.** Astrocytes were incubated with fresh Aβ-(1–42) (*panel A*) and aged Aβ-(1–42) (*panel B*) for different time periods (30, 60, 90, and 120 min) after which time cells were harvested as described under "Experimental Procedures." Cells were placed in 96-well plates, and PC-PLD was determined using the Amplex Red PC-specific PLD assay kit (Molecular Probes). Fluorescence intensity was measured with a microplate reader using an excitation wavelength of 542 nm and an emission wavelength of 590 nm. Values are means ± S.E. ($n = 3$). *, $p < 0.001$ as compared with control cells; +, $p < 0.001$ as compared with 30-min incubation time.

DISCUSSION

Several different lines of evidence show that Aβ and cholesterol interact (for reviews, see Refs. 4 and 44–47). This interaction is reciprocal given that cholesterol modulates expression of amyloid precursor protein and Aβ (10–13) and cholesterol dynamics such as cholesterol trafficking are affected by Aβ (15, 17–19). The Golgi complex plays an important role in cholesterol trafficking (21, 48), and we proposed that Aβ-(1–42) would alter cholesterol trafficking in the Golgi complex and that the effects of Aβ-(1–42) would be dependent on whether the peptide was fresh or aged. Two different methods, confocal microscopy and isolation of the Golgi complex by sucrose density centrifugation, were used to test the hypothesis that fresh and aged Aβ-(1–42) would have different effects on cholesterol in the Golgi complex of astrocytes. Results of this study showed

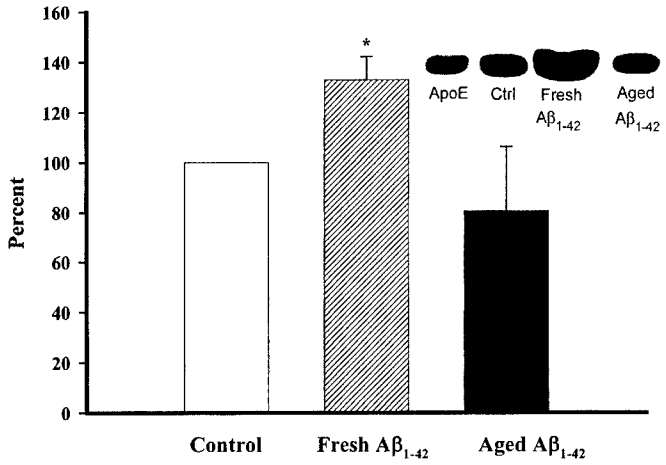

FIG. 5. **Fresh Aβ-(1–42) but not aged Aβ-(1–42) increases apoE levels in astrocytes.** Cells were treated as described under "Experimental Procedures." Levels of apoE were quantitated from Western blots. Data of cells treated with Aβ-(1–42) are the levels of apoE relative to control cells and are the means ± S.E. from three to four independent experiments. The *inset* shows a Western blot from a representative sample. *, $p \leq 0.01$ as compared with control. *Ctrl*, control.

that Aβ-(1–42) modifies cholesterol distribution in the Golgi complex of astrocytes. However, the effects of Aβ-(1–42) on cholesterol distribution were dependent on whether Aβ-(1–42) was fresh or aged. Fresh Aβ-(1–42) increased cholesterol content in the Golgi complex, and aged Aβ-(1–42) reduced cholesterol content in the Golgi complex. Moreover isolation of the Golgi complex into two fractions revealed the novel finding that fresh Aβ-(1–42) increased cholesterol in the G1 fraction and reduced cholesterol in the G2 fraction. Aged Aβ-(1–42) reduced cholesterol in both fractions. PC-PLD activity, cell membrane cholesterol, and apoE levels were associated with effects of fresh Aβ-(1–42) on cholesterol levels but not with effects of aged Aβ-(1–42), arguing against a common mechanism.

Aβ-(1–42) altered cholesterol distribution in the Golgi complex of astrocytes. Maximal effects of both fresh and aged Aβ-(1–42) were observed after 120 min of incubation and a peptide concentration of 10^{-6} M. Shorter incubation times modified cholesterol levels in the Golgi complex, but these effects were not significantly different. An Aβ-(1–42) concentration of 10^{-9} M did not modify cholesterol levels. Aβ-(1–42)-induced changes in Golgi complex cholesterol levels were not simply attributable to a nonspecific perturbation of the Golgi complex or cell viability. The reverse peptide Aβ-(42–1), either fresh or aged, did not alter Golgi complex cholesterol levels compared with control astrocytes. Viability of cells did not differ among the control and cells treated with fresh or aged Aβ-(1–42).

Fresh Aβ-(1–42) increased astrocyte cholesterol in the G1 fraction by 59% but reduced cholesterol in the G2 fraction by 29%. Earlier work has indicated that the G1 fraction may represent the *trans*-Golgi region and that the G2 fraction is representative of the *medial* and *cis* regions (31, 32). Fresh Aβ-(1–42) may stimulate movement of cholesterol from the *cis* and *medial* regions of the Golgi to the *trans*-Golgi region. Mechanisms of anterograde intra-Golgi transport have focused on cisternal maturation/progression and vesicular/tubular transport, but the principal mechanism of intra-Golgi transport including transport of lipids has not been resolved (for a review, see Ref. 49). Movement of cholesterol from the G2 to the G1 fraction induced by fresh Aβ-(1–42) would account for only 29% of the increase in cholesterol observed in the G1 fraction. Our data showing that there was a significant reduction in cholesterol content in the cell membrane of astrocytes treated with fresh Aβ-(1–42) (34%) as compared with control astrocytes suggest that fresh Aβ-(1–42) stimulates transport of cholesterol from the cell membrane to the *trans*-Golgi region. There is evidence that cycling of lipids including cholesterol and proteins occurs between the cell surface membrane and the Golgi complex that is clathrin-independent (50, 51). It has been suggested that this cell membrane-Golgi pathway may regulate lipid raft distribution and function with recycling occurring continuously (51). Amyloid precursor protein and Aβ are associated with cholesterol-rich low density membrane domains (52–55). Extracellular Aβ that is not highly aggregated may target lipid rafts, promoting cycling of lipid rafts to the Golgi complex.

Aged Aβ-(1–42) reduced cholesterol levels in the Golgi complex but also reduced cholesterol levels in the cell membrane by 16% as compared with the 34% reduction by fresh Aβ-(1–42). The destinations and mechanisms of the cell membrane cholesterol removed by fresh and aged Aβ-(1–42) may differ. Aged Aβ-(1–42) may complex with cholesterol in the cell membrane and transport cholesterol to the cell interior. Support for this notion are data showing that indeed aged Aβ-(1–40), but not fresh Aβ-(1–40), complexed with lipids with cholesterol ≫ stearic acid > PC (27). Once removed, the aged Aβ-(1–42)-cholesterol complex may act as a type of lipid droplet in the cell cytosol. Synucleins that had been chemically cross-linked formed oligomers within cells that complexed with lipid droplets and cell membranes (56). It is clear that fresh Aβ-(1–42) and aged Aβ-(1–42) are acting differently on cholesterol homeostasis.

Cholesterol levels were increased in the Golgi complex by incubation with fresh Aβ-(1–42), and this increase may be due in part to cycling of cholesterol from the cell membrane to the Golgi complex. We also propose that the elevation in cholesterol levels in the Golgi complex involves PC-PLD function. PC-PLD plays a role in cholesterol trafficking, it is located in the Golgi complex, and PC-PLD is also involved in vesicular trafficking from the Golgi complex (20–23). Our findings that fresh Aβ-(1–42) increased cholesterol content in the Golgi complex and inhibited activity of PC-PLD are in agreement with the purported role of PC-PLD in cholesterol trafficking. Inhibition of PC-PLD reduces cholesterol efflux (22, 42). Activation of PC-PLD enhanced the release of secretory vesicles from the *trans*-Golgi complex (43). Inhibition of PC-PLD by fresh Aβ-(1–42) may in turn reduce release of secretory vesicles with their lipid and protein cargoes. We found that fresh Aβ-(1–42) inhibited PC-PLD activity, but another study reported that the Aβ peptide fragment 25–35 increased PLD activity in LA-N-2 cells (57). Differences in effects of Aβ on PLD activity between that study and the present study may have resulted from dissimilarities in peptide structure and peptide concentration (Aβ-(1–42) at 1 μM *versus* Aβ-(25–35) at 100 μM). Inhibition of PC-PLD activity by fresh Aβ-(1–42) was in agreement with our findings that cholesterol levels were elevated in the Golgi complex in the presence of fresh Aβ-(1–42). However, we also observed that aged Aβ-(1–42) significantly inhibited PC-PLD activity but that cholesterol levels were reduced in the Golgi complex of astrocytes incubated with aged Aβ-(1–42). Both fresh and aged Aβ-(1–42) inhibit PC-PLD activity, but the mechanism of effects on cholesterol levels in the Golgi complex may differ as discussed in the preceding paragraph. Aged Aβ-(1–42) may remove cholesterol by forming complexes, whereas fresh Aβ-(1–42) induces movement of cholesterol within the Golgi complex and from the cell membrane to the Golgi complex and inhibits the recycling of cholesterol that involves PC-PLD function.

The effects of fresh Aβ-(1–42) on Golgi complex cholesterol were associated with apoE levels. Fresh Aβ-(1–42) signifi-

cantly increased apoE levels in astrocytes by ~32% as compared with control cells. On the other hand, aged Aβ-(1–42) reduced apoE levels, but this reduction did not differ significantly from that seen in control cells. Previously it was found that aged Aβ-(1–42) increased apoE levels in rat astrocytes from primary cell culture (18). In the same study it also was reported that aged Aβ-(1–42) reduced levels of apoE in the medium (18). We did not detect apoE in the media of control astrocytes and cells incubated with either fresh or aged Aβ-(1–42). Two major differences between our study and the earlier report were the incubation time of 2 *versus* 12 h and the Aβ-(1–42) concentration of 1 *versus* 10 μM, respectively. An important observation of the earlier study was that Aβ-(1–42) did not alter steady-state levels of apoE mRNA, and that may indicate that Aβ-(1–42) was not acting on transcriptional regulation but could have been acting post-translationally on apoE turnover (18). Whether the observed increase in apoE levels in astrocytes incubated with fresh Aβ-(1–42) plays a role in effects of the peptide on cholesterol in the Golgi complex is unclear. ApoE is associated with the Golgi complex (58), and changes in apoE levels may modify intracellular cholesterol distribution.

We have shown using two different methods that Aβ-(1–42) modifies the cholesterol content of the Golgi complex of astrocytes. The effects of Aβ-(1–42) on cholesterol levels were dependent on whether astrocytes were treated with fresh or aged Aβ-(1–42). Fresh Aβ-(1–42) increased cholesterol levels of the Golgi complex, whereas aged Aβ-(1–42) reduced cholesterol levels. However, the direction of effects of fresh Aβ-(1–42) on Golgi cholesterol levels was contingent on the region of the Golgi complex suggesting that fresh Aβ-(1–42) may also be acting on intra-Golgi cholesterol transport. The Golgi complex has been described as a major site for protein and lipid sorting and release of lipid rafts (59, 60). Either increasing or decreasing cholesterol levels could impact on important Golgi complex functions such as protein sorting, sphingomyelin synthesis, and lipid trafficking resulting in cellular pathophysiology. Our data suggest that the recycling of cholesterol between the cell membrane and the Golgi complex was altered and that such changes could modify membrane structure and function. Fresh Aβ may inhibit the formation of lipid rafts in the Golgi complex including caveolin-organized rafts that are transported to the plasma membrane and form caveolae. Aβ peptides are located in detergent-insoluble membrane domains that are thought to be caveolae (54, 61) and in cholesterol domains not containing caveolin (52). PC-PLD activity, cell membrane cholesterol, and apoE expression of astrocytes were associated with effects of fresh Aβ-(1–42) on Golgi complex cholesterol but not with effects of aged Aβ-(1–42), arguing against a common mechanism. There is a reciprocal synergy between Aβ and cholesterol. Changes in cholesterol content alter Aβ levels, and Aβ acts on cholesterol homeostasis. The dynamic interaction of Aβ and cholesterol that includes targeting of the Golgi complex may be an important factor that contributes to cellular dysfunction occurring with AD.

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