Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts

Bassam Haidar,[1] Stephanie Mott,[1] Betsie Boucher, Ching Yin Lee, Michel Marcil, and Jacques Genest, Jr.[2]

Cardiovascular Genetics Laboratory, McGill University Health Center, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1

Abstract **Familial HDL deficiency (FHD) is the heterozygous form of Tangier disease (TD). Mutations of the ABCA1 gene cause FHD and TD. FHD/TD cells are unable to normally efflux cholesterol onto nascent HDL particles, which are rapidly catabolized. TD fibroblasts have an abnormal pattern of PLC and PLD activation following cell stimulation with HDL_3 or apolipoprotein A-I (apoA-I). We examined cellular cholesterol efflux in FHD and TD fibroblasts by phospholipid-derived-molecules, compared with that of normal cells. We used the PKC agonist 1,2-dioctanoylglycerol (DOG) and phorbol myristate acetate (PMA) to activate PKC, calphostin C, and GÖ 6976, as inhibitors of PKC; phosphatidic acid (PA), which is the product of PLD, and lysophosphatidic acid (LPA), phosphatidylcholine, sphingomyelin, and β-cyclodextrin to investigate their potential effect in modulating cellular cholesterol efflux in [^{3}H]cholesterol-labeled and cholesterol-loaded fibroblasts. Phosphatidylcholine, sphingomyelin, and β-cyclodextrin promoted cholesterol efflux in an identical fashion in control, FHD, or TD fibroblasts. In a dose-dependent fashion, DOG (0–200 μM) increased apoA-I-mediated cellular cholesterol efflux by 40% in controls, 71% in FHD, and 242% in TD cells. PMA similarly increased cholesterol efflux to a maximum of 256% in controls, 182% in FHD, and 191% in TD cells. This effect was inhibited by calphostin C. PA (100 μM) also increased cholesterol efflux by 25% in control, 44% in FHD, and 100% in TD cells. Conversely, LPA reduced cholesterol efflux in a dose-dependent fashion in control and FHD cells (−50%, 200 μM) but not in TD cells, where efflux was increased by 140%. Propranolol (100 μM) significantly increased cholesterol efflux at 24 h in all three cell lines. *n*-Butanol partially decreased the DOG-mediated increase in cholesterol efflux. The inhibitory effect of calphostin C on DOG-stimulated cholesterol efflux could be partially overcome by propranolol, suggesting that PA is a downstream mediator of PKC-stimulated cholesterol efflux. We conclude that PLC and PLD activities are required for apoA-I-mediated cellular cholesterol efflux, and modulating cellular PA concentration can correct, at least partially, the cholesterol efflux defect in FHD and TD.**—Haidar, B., S. Mott, B. Boucher, C. Y. Lee, M. Marcil, and J. Genest, Jr. **Cellular cholesterol efflux is modulated by phospholipid-derived signaling molecules in familial HDL deficiency/Tangier disease fibroblasts.** ***J. Lipid Res.*** **2001.** 42: **249–257.**

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High density lipoproteins (HDL) are involved in cholesterol transport and contribute to a remarkably large spectrum of biological processes (1, 2). Epidemiological studies have consistently shown a reverse association between HDL-cholesterol (HDL-C) levels and the presence of coronary artery disease (CAD) (3–10). Most cases of reduced HDL-C levels are secondary to increased secretion of apolipoprotein (apo)B-containing lipoproteins of hepatic origin (11). Isolated HDL deficiency is found in approximately 4% of premature CAD subjects (11). Rare causes of HDL deficiency include apoA-I abnormalities, disorders of HDL processing enzymes, and familial HDL deficiency (FHD) or Tangier disease (TD). FHD is defined as a HDL-C level <5th percentile for age- and gender-matched subjects (12) and is caused by a defect in cellular cholesterol efflux onto nascent HDL particles. Decreased apoA-I- or HDL_3-mediated cellular cholesterol efflux in skin fibroblasts is observed in FHD patients (13). A similar defect has also been reported in cells from TD subjects (14–16) (although more severe) and in patients with severe HDL deficiency (17). The reduced cellular choles-

Abbreviations: ABCA1, ATP binding cassette-1; apo, apolipoprotein; DAG, diacylglycerol; DOG, 1,2-dioctanoylglycerol; FHD, familial HDL deficiency; HDL, high density lipoprotein; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PKC, protein kinase C; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol myristate acetate; SM, sphingomyelin; TD, Tangier disease.

[1] Both first authors contributed equally to this work.

[2] To whom correspondence should be addressed.
e-mail: jacques.genest@muhc.mcgill.ca

terol efflux results in the inability or inefficiency to provide cholesterol to nascent HDL particles, leading to a marked decrease in the formation of spherical cholesteryl ester-rich lipoproteins with α-migrating properties on agarose gel electrophoresis (13). Kinetic studies on HDL metabolism with endogenously labeled apoA-I-containing particles show that these particles are rapidly catabolized (18). We have recently shown that FHD and TD are caused by mutations in the adenosine triphosphate (ATP)-binding cassette-1 (ABCA1) gene on chromosome 9q31 (19, 20). FHD is the heterozygous form of TD. The ABCA1 gene encodes a member of the ATP-binding transporter superfamily, which is involved in energy-dependent transport of many substrates across membranes. Other members of this superfamily are the multidrug resistant proteins and the p-glycoproteins that are driven by ATP hydrolysis (21, 22).

Studies performed on human skin fibroblasts show that cellular cholesterol transport and efflux are, in large part, mediated by HDL particles (and other acceptors) in two separate processes (20, 23): first, by passive diffusion of membrane-associated cholesterol and phospholipids onto apoA-I-containing lipoproteins or phospholipid vesicles, and second, by a process in which apoA-I and apoA-I-containing lipoprotein-cell interaction promotes the hydrolysis of stored cellular cholesteryl esters and subsequent translocation of cholesterol to the plasma membrane. This process can be inhibited by brefeldin A and monensin, which disrupt the *trans*-Golgi network (24). Although the precise mechanisms of lipoprotein-cell interaction are unclear and, to date, a specific apoA-I or HDL receptor mediating this effect has not yet been characterized (25), a model has been proposed for cellular cholesterol efflux (23). ApoA-I (or HDL_3) mediates the activation of phosphatidylcholine (PC)-specific phospholipases C and D (PLC and PLD, respecitively) and the hydrolysis of membrane phospholipids with the generation of diacylglycerol (DAG) and phosphatidic acid (PA), respectively. This response is postulated to be mediated by a G-protein-coupled receptor and activates protein kinase C (PKC) (26). By mechanisms still poorly understood, cholesterol translocation to the plasma membrane is stimulated.

To determine whether the defective cholesterol translocation and efflux pathways could be modulated in vitro, the objective of the present study was to examine the effects of 1,2-dioctanoylglycerol (DOG), phorbol myristate acetate (PMA), PA, lysophosphatidic acid (LPA), sphingomyelin (SM), PC, β-cyclodextrin, and lysophosphatidylcholine (LPC) on cellular cholesterol efflux.

METHODS

Cell culture medium, supplements, and fetal bovine serum (FBS) were obtained from Gibco BRL. [^{3}H]cholesterol was obtained from Mandel. Cholesterol, DOG, PA, LPA, SM, PC, LPC, β-cyclodextrin, *n*-butanol, and propranolol were purchased from Sigma, and PMA, calphostin C, DAG kinase inhibitor II, and GÖ 6976 from Calbiochem.

Patient selection

Patients with FHD were selected as previously described (12, 13). Five probands from four different kindred with FHD, control subjects, and one patient with TD have been previously characterized (20, 27). Briefly, FHD subjects are defined as having a very low HDL-C (<5th percentile for age and gender matched, with triglycerides <95th percentile) transmitted as an autosomal codominant trait associated with a marked reduction of HDL_3- and apoA-I-mediated cellular cholesterol efflux and the absence of the clinical signs of TD. The biochemical characteristics of these subjects are detailed elsewhere (20, 27). For the present study, we selected fibroblasts from one normal control subject, one patient with FHD (ABE), and one TD patient. Analysis of ABCA1 gene revealed a compound heterozygote state for the TD subject. The FHD patient has been previously referred to as 24430-301 (ABE) and the TD patient as TD1 in Mott et al. (27). The protocol for the study was reviewed by the Research Ethics Board of the McGill University Health Center. All study subjects and family members signed separate informed consent forms for plasma sampling, analysis and storage, DNA analysis and storage, and skin biopsy.

Cell culture

Human skin fibroblasts were obtained from 3.0-mm punch biopsies of the forearm of patients and healthy control subjects and were cultured as described (27). We seeded 5×10^4 cells in 35-mm cell culture wells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. At approximately 50% confluence, 0.2 μCi [^{3}H]cholesterol/ml was added in the cell medium. When the cells reached confluence, they were washed three times in phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA), and the medium was replaced by DMEM without serum (cells in growth arrest) containing 2 mg/ml BSA and 20 μg/ml of free non-lipoprotein cholesterol for 24 h. Cellular cholesterol pools were allowed to equilibrate for another 24 h in DMEM containing 1 mg/ml BSA. Efflux studies (0–24 h) were then performed in the presence and absence of specified efflux-modulating signaling molecules and 10 μg/ml of purified apoA-I. The cell culture media used in efflux studies was DMEM without serum and 1 mg/ml BSA unless specified. All lipid products were dissolved as recommended by the manufacturer [PA, LPA, and PC were dissolved in chloroform; SM in chloroform–methanol 1:1 (v/v); LPC was dissolved directly in Tris buffer, evaporated under a stream of nitrogen, and resuspended in a Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.00)] before being added to the efflux medium. Although the highest concentrations used in the experiments never exceeded 2%, Tris buffer showed no influence on cholesterol efflux at concentrations of up to 10% (data not shown).

Modulation of the PKC, PLD, and PLC pathways

Figure 1 summarizes metabolic pathways of PC hydrolysis and shows the sites of action for PLC and PLD, as well as phospholipase A_2. Propranolol (100 μM) was used as a specific inhibitor of PA-phosphohydrolase (PAPH); and the conversion of DAG to PA was also inhibited by DAG kinase inhibitor II (0.1–10 μM). DOG (a DAG analog) and PMA at a concentration of 160 nM were used as stimulators of PKC. Calphostin C was used as a selective inhibitor of the PKC regulatory site. The calcium-dependent PKC inhibitor GÖ 6976 was used at concentrations of 0.10–5.0 μM for 24 h with apoA-I. PA was used in concentrations from 0–150 μM. At concentrations >150 μM for PA and >200 μM for DOG, cell toxicity was observed with cell death and reduced cellular protein concentration in culture wells. Cells treated with

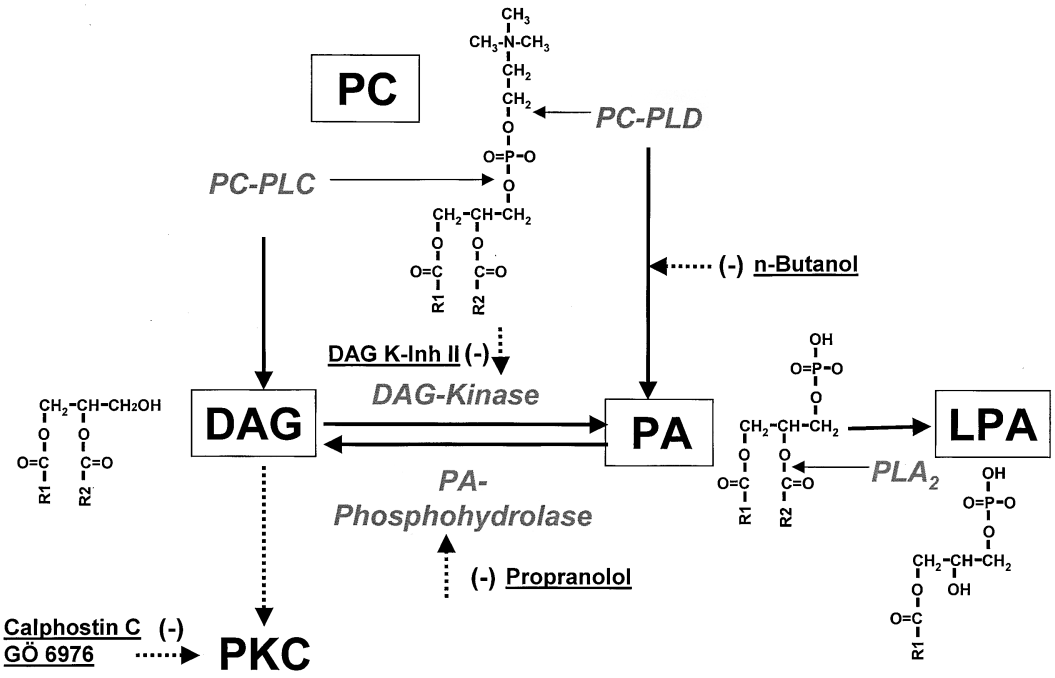

Fig. 1. Phospholipid pathways. Phospholipids are composed of a backbone of glycerol, with two fatty acyl chains in positions R1 and R2. R3 is occupied by a phosphate group to which ethanolamine (phosphatidyl ethanolamine), choline (PC), serine (phosphatidyl serine), or inositol (phosphatidyl inositol) is attached. PLA_1 cleaves the first R1 fatty acyl chain and PLA_2 cleaves the second, generating a lysophospholipid. PC-PLC generates DAG and choline phosphate, and PLD generates PA and choline (thin arrows). DAG can be phosphorylated to PA by DAG kinase, and PA can be converted to DAG by PA phosphohydrolase. This last step can be inhibited by propranolol. The formation of PA from PC through the action of PLD can be reduced by using *n*-butanol as a substrate for PLD, yielding phosphobutanol. The combined actions of PLA_2 and PLD generate LPA. Calphostin C and GÖ 6976 inhibit PKC. DAG K-Inh, diacylglycerol kinase inhibitor II. Inhibitors are indicated by a (−) sign.

calphostin C (50 nM) were exposed to fluorescent light for 1 h at 37°C for product activation. Because there are currently no commercially available specific PC-PLD inhibitors, formation of PA by PLD was decreased by the use of *n*-butanol, a substrate for PLD. Through transphosphatidylation, *n*-butanol is converted to phosphobutanol (Pbut), and thus decreases the formation of PA. The efficiency of this pathway was estimated at 17% in normal and 33% in TD fibroblasts (26). We also used LPA (0–250 μM), which has been shown to modulate cellular effects through a specific receptor (28).

Cellular cholesterol efflux determination

ApoA-I-mediated (10 μg/ml) cholesterol efflux was carried out on the three cell lines; as control, we used fatty acid-free BSA only in the efflux medium. All experiments were performed in triplicate. Efflux was determined at the specified time points from 0 to 24 h. At the indicated time, cells were chilled on ice and the medium was collected. The cell layer was harvested overnight in 0.1 N NaOH at 4°C. Cellular protein concentration was determined according to Lowry et al. (29) and one 150-μl aliquot was reserved for scintillation counting of cellular [^{3}H]cholesterol content. Cellular cholesterol efflux was determined as follows: ^{3}H in medium/(^{3}H in medium + ^{3}H in cells); the results were expressed as percentage of total [^{3}H]cholesterol. Data were also analyzed after correction for cell proteins. The efflux results were similar for both corrected and uncorrected data. In control cells, 8–12% of total [^{3}H]cholesterol was found in the medium after stimulation with apoA-I for 24 h. Control experiments with DMEM + 0.1% BSA resulted in 1–2% cellular cholesterol efflux.

Paired two-tailed Student's *t*-tests were used to compare values of cholesterol efflux under various pharmacological manipulations between FHD, TD, and control cell lines. A *P*-value < 0.05 was considered statistically significant. The data are presented as mean ± standard deviation unless specified.

ApoA-I preparation

HDL was isolated from fresh plasma of normolipidemic donors using standard sequential ultracentrifugation with density (1.125–1.210 g/ml) adjusted with KBr. The HDL preparation was delipidated in acetone–ethanol 1:1 (v/v) and total proteins were fractionated at 4°C on two Sephacryl S-200 (Pharmacia) columns (2.6 × 100 cm). Fractions contained in the apoA-I peak were extensively dialyzed in 0.01 M NH_4HCO_3, lyophilized, and resuspended in PBS at a concentration of 1 mg/ml. Protein purity was assayed on polyacrylamide gradient gel electrophoresis for each apoA-I fraction and appropriate fractions were pooled, dialyzed in PBS, and lyophilized before being stored at −70°C as stock (13).

RESULTS

The cholesterol efflux defect observed in FHD has been reported elsewhere (13, 27). For the present study, we selected one of five FHD subjects from four families in whom a cholesterol efflux defect was identified (27). Compared with skin fibroblasts from normal subjects, the

Table 1. Lipid values of control, FHD, and TD subjects

Subject	ID	Age	Gender	Total C	HDL-C	ApoA-I	TG	LDL-C
				mmol/l	*mmol/l*	*mg/dl*	*mmol/l*	*mmol/l*
CTR	JGE	40	M	5.80	1.63	120	2.00	3.25
FHD	ABE	53	M	3.68	0.18	25	1.48	3.04
TD	TD	42	M	2.30	<0.1	2	1.96	1.39

C, cholesterol; TG, triglycerides; CTR, control; LDL, low density lipoprotein; M, male.

mean reduction in apoA-I-mediated (10 μg/ml) cholesterol efflux from those five subjects was 59%. The selected proband for this study had the most severe efflux defect of the FHD probands tested, which was decreased by 55% of control values. In contrast, cells from a subject with TD had a ~90% reduction in apoA-I-mediated efflux. Each of the experiments was performed on fibroblasts from one FHD, one TD patient, and one normal control. The characteristics of these three subjects are shown in **Table 1**. The TD patient, referred to as TD1 (19), had a mutation in ABCA1 T4369C leading to a Cys1417Leu substitution in exon 30 and a splice site mutation in exon 24, leading to a truncated mRNA species. The patient with FHD (ABE) (13) had a mutation in exon 48 C6370T, resulting in a Arg2084STOP nonsense codon and a predicted loss of the terminal 118 amino acids of the ABCA1 gene product (20). No other mutations of the ABCA1 gene were identified in this subject.

In the present study, we examined the effect of phospholipid-derived molecules on cellular cholesterol efflux (Fig. 1). To determine whether passive diffusion of cellular cholesterol differed in control, FHD, and TD cells, we examined the 24-h cholesterol efflux onto phospholipids (PC, 100 μM; SM, 100 μM) and the 12-h efflux for β-cyclodextrin (1 mM). These experiments were performed in DMEM with appropriate concentrations of acceptor molecules and in the absence of HDL or apolipoproteins in the medium. We found that cellular cholesterol efflux in the presence of these acceptors was identical in the three cell lines (**Fig. 2**), suggesting that passive desorption of cellular cholesterol was not affected by mutations at the ABCA1 gene locus.

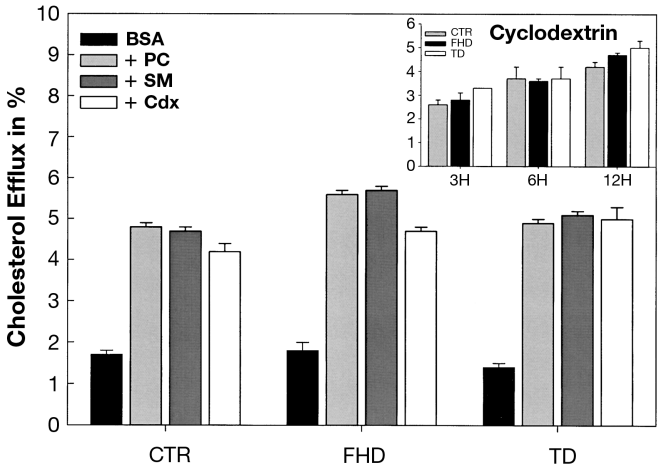

Fig. 2. Effect of equimolar amounts (100 μM) of SM, PC, sphingosine (Sph), and β-cyclodextrin (Cdx, 1 mM) on cellular cholesterol efflux. Cells were grown in Dulbecco's modified Eagle's medium/fetal bovine serum and labeled with [^{3}H]cholesterol (0.2 μCi/ml), then loaded with cholesterol (20 μg/ml) in growth arrest. The efflux medium contained only bovine serum albumin (BSA) as cholesterol acceptor. The 24-h efflux with PC and SM was identical for control (CTR), FHD, and TD, suggesting that membrane-associated cholesterol desorbed normally in FHD and TD cells. Cholesterol efflux, at 3, 6, and 12 h for Cdx was similar for the three cell lines (inset).

The effect of PC and SM on apoA-I-mediated cellular cholesterol efflux was then tested. In the presence of apoA-I, there was a significant increase in cholesterol efflux with PC [+101% in control (9.1 ± 0.5% vs. 18.3 ± 0.2%, $P = 0.001$); +111% in FHD (7.4 ± 0.5% vs. 15.6 ± 0.4%, $P = 0.001$); and +229% in TD cells (1.7 ± 0.1% vs. 5.6 ± 0.3%, $P = 0.00$; **Fig. 3**)]. Furthermore, there was an even more pronounced effect with SM in a dose-dependent fashion at 0, 10, 50, and 100 μM (Fig. 3, inset), where cholesterol efflux was increased by 189% in control (9.1 ± 0.5% vs. 26.3 ± 0.6%, $P = 0.001$), 231% in FHD (7.4 ± 0.5% vs. 24.5 ± 0.9%, $P < 0.001$), and 670% in TD cells (1.7 ± 0.1% vs. 13.1 ± 0.3%, $P < 0.001$; Fig. 3).

The potential role of PKC in cholesterol efflux has been postulated in previous studies (30, 31). The effect of DOG (a PKC agonist and short-chain analog of DAG) on cellular cholesterol efflux was examined in a dose-dependent fashion; results of normal, FHD, and TD cells are shown in **Fig. 4**. We used apoA-I (10 μg/ml in medium) as cholesterol acceptor because differences in efflux between the three cell lines were more pronounced compared with HDL_3 (13). At doses of 0–200 μM of DOG, cellular cholesterol efflux at 24 h increased in a dose-dependent fashion. At a maximal concentration of 200 μM, cholesterol efflux increased by 40% in controls (14.0 ± 0.3% vs. 19.6 ± 1.6%, $P < 0.05$), 71% in FHD (7.3 ± 0.07% vs. 12.5 ± 1.09%, $P < 0.05$), and 242% in TD cells (2.6 ± 0.4% vs. 8.9 ± 0.5%, $P < 0.01$). Similar results were found for the 12-h time point (data not shown). To strengthen the concept that PKCs are involved in cellular cholesterol efflux, we used PMA (160 nM, 3-h preincubation) to activate PKC, and measured ^{3}H cellular cholesterol efflux at 24 h. As shown in **Fig. 5**, PMA significantly increased cholesterol efflux in controls (256%, $P < 0.001$), FHD (182%, $P < 0.001$), and TD cells (191%, $P = 0.005$).

The effect of PA was then investigated with doses varying from 0–150 μM. At a concentration of 100 μM, PA, in the presence of apoA-I, increased cholesterol efflux in control cells to a maximum of 125% ($P = 0.015$) and to 144% in FHD cells ($P = 0.018$), compared with apoA-I alone. In TD cells, efflux was increased to 200% of control percentage of basal levels at 100 μM PA ($P < 0.05$; **Fig. 6**). Similar results were observed at the 12-h time point (data not shown). At a concentration of 200 μM, PA decreased efflux significantly in all three cell lines. Thus, PA exerted a significant increase in cholesterol efflux in TD cells. The PAPH inhibitor propranolol (100 μM) was used to decrease the conversion of PA to DAG. In the presence of apoA-I for 24 h, propranolol increased cholesterol efflux by

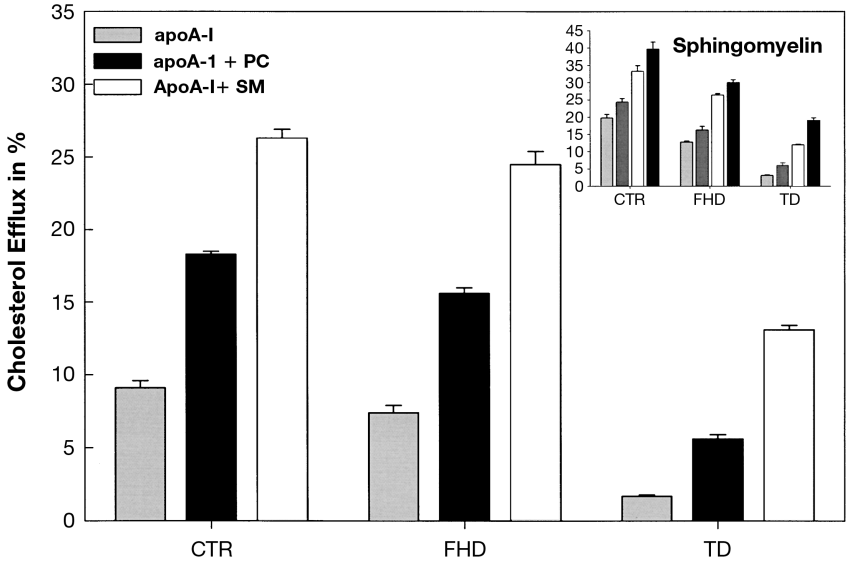

Fig. 3. ApoA-I-mediated cellular cholesterol efflux in the presence of equimolar amounts (100 μM) of SM and PC. A marked increase in cholesterol efflux was noted for apoA-I + PC and for apoA-I + SM. Inset: Dose-dependent increase in cholesterol efflux with SM (0, 1, 10, and 100 μmol/l) after cell stimulation with apoA-I (10 μg/ml) for 24 h. CTR, control.

35% in controls (10.7 ± 1.44% vs. 14.3 ± 1.0%, $P = 0.08$), 45% in FHD (7.7 ± 0.6% vs. 11.0 ± 0.5%, $P < 0.05$), and 59% in TD cells (2.6 ± 0.6% vs. 4.3 ± 0.7%, $P < 0.05$; Fig. 6, inset). We also examined the effect of *n*-butanol, which is used by PLD in a transphosphorylation reaction resulting in decreased formation of PA from PC. To further investigate the hypothesis that PKC activates PLD and subsequently stimulates the production of PA, the cells were stimulated with DOG (150 μM) as described above with or without *n*-butanol. In the presence of *n*-butanol (0.3%), cholesterol efflux stimulated by DOG and apoA-I was reduced by 30% in controls (18.0 ± 0.6% vs. 12.6 ± 0.6%, $P = 0.001$), 39% in FHD (6.2 ± 0.3% vs. 3.7 ± 0.8%, $P = 0.056$, not significant), and 16% in TD cells (5.1 ± 0.06% vs. 4.3 ± 0.15%, $P < 0.05$) at the 12-h time point (data not shown). We also inhibited the conversion of DAG to PA by DAG kinase inhibitor II (0.1–10 μM) and did not find a significant change in cellular cholesterol efflux (data not shown). Taken together, these data strengthen the concept that DOG activated PKC, which in turn activated PC-PLD, producing PA, which is required for cellular cholesterol translocation and efflux.

We used the PKC antagonist calphostin C (50 nM) in efflux experiments mediated by apoA-I and DOG. As shown in **Fig. 7**, calphostin C caused a marked decrease (−50%; $P < 0.001$) in apoA-I-mediated cellular cholesterol efflux at 12 h and an even greater (−70%; $P < 0.001$) decrease in DOG-mediated efflux at 12 h. The calcium-dependent PKC inhibitor GÖ 6976 (5 μM) yielded similar results, with an inhibition in apoAI- and DOG-mediated cellular cholesterol efflux of 60% ($P < 0.001$) and 57% ($P < 0.001$), respectively (data not shown). These data lend

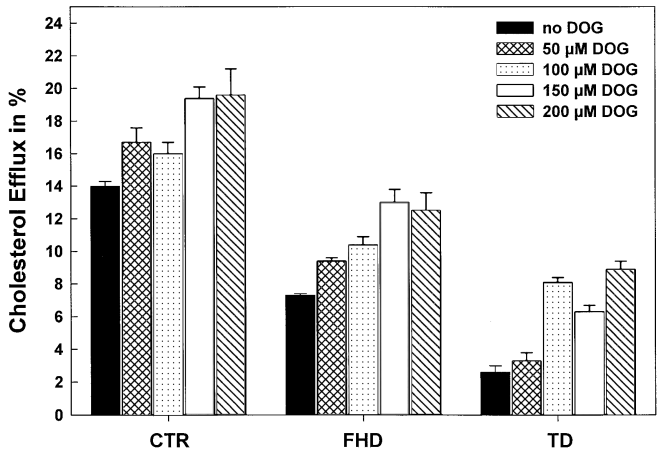

Fig. 4. Effect of DOG (0–200 μM) on cellular cholesterol efflux. DOG was added to the culture medium and efflux was determined after 24 h. CTR, control.

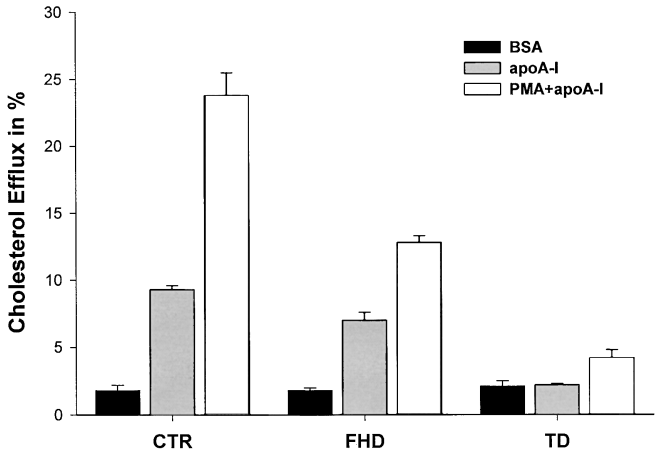

Fig. 5. Effect of PMA (160 nmol/l) on apoA-I-mediated cellular cholesterol efflux. Cells were preincubated with PMA for 3 h and apoA-I-mediated efflux was performed at 24 h. CTR, control.

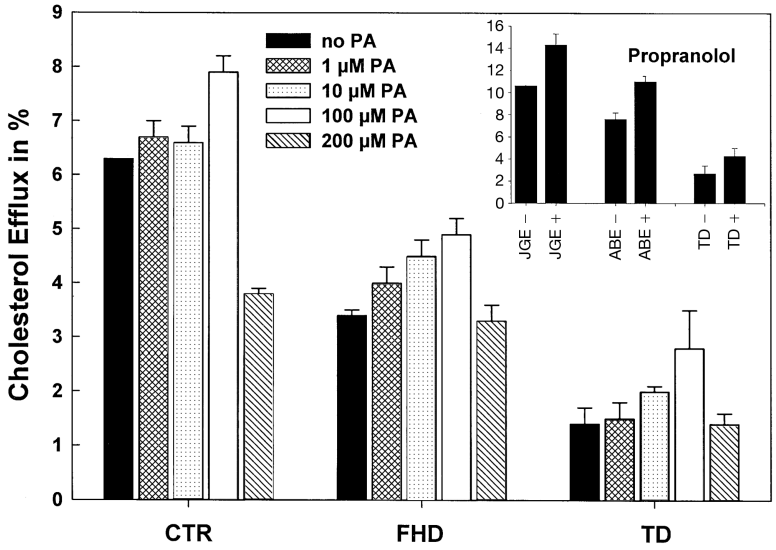

Fig. 6. PA increased cholesterol efflux in control (CTR), FHD, and TD cells in a dose-dependent fashion (0–150 μmol/l). To increase PA availability, the same cells were used in the absence (−) or presence (+) of propranolol (100 μM) to inhibit PA phosphohydrolase. Inset: Propranolol increased apoA-I-mediated cellular cholesterol efflux in controls (JGE), FHD (ABE), and TD cells.

support to the concept that fibroblasts PKC isoforms are involved in cellular cholesterol efflux. Subsequently, we used the PAPH inhibitor propranolol in the experiments where calphostin C and DOG were used. Cellular cholesterol efflux was partially corrected by propranolol (100 μM), suggesting that both DAG (an activator of PKC) and PA (32) are generated after apoA-I stimulation and are required for cholesterol efflux.

As PA can be converted in cells to LPA by PLA_2 or obtained from the action of PLD on LPC, the effects of this phospholipid on cellular cholesterol efflux was examined as well. At concentrations between 0 and 200 μM, LPA, in contrast to PA, showed a reduced cholesterol efflux in control and FHD cells, but not in TD cells where efflux was increased in a dose-dependent fashion. At a concentration of 200 μM, LPA decreased efflux in control cells by 50% (10.7 ± 0.9% vs. 5.4 ± 0.8%, $P < 0.05$) and in FHD cells by 47% (7.0 ± 0.6% vs. 3.7 ± 0.2%, $P < 0.05$), but increased efflux in TD cells by 140% (2.5 ± 0.5% vs. 6.0 ± 0.5%, $P < 0.00$; **Fig. 8**). A similar pattern was observed at the 12-h time point (data not shown). These experiments were performed four times, with similar results.

When cells were stimulated with DOG, PMA, or PA, but without an acceptor particle (apoA-I or HDL_3) in the culture medium (other than BSA), no significant increase in medium [^{3}H]cholesterol was found.

The effects of LPC (100 μM) was also examined. Because of its strong detergent properties (33), it is cytolytic at this concentration (data not shown). We were unable to document a physiological effect of LPC on cholesterol efflux at the concentration used.

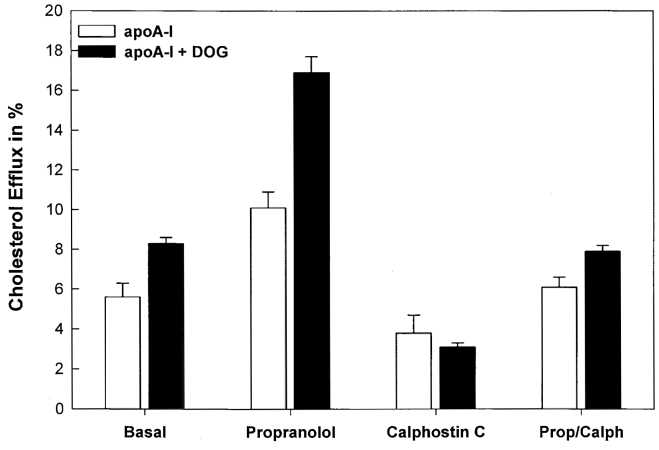

Fig. 7. Twelve-hour cholesterol efflux in control cells in the presence of apoA-I (open bars) and apoA-I with DOG (black bars) with addition of propranolol (150 μM), calphostin C (50 nM), or both. Calphostin C markedly inhibited apoA-I- and DOG-stimulated cholesterol efflux. This effect was partly reversed by propranolol, suggesting that PA production mediates, at least in part, the efflux pathway.

DISCUSSION

Since its original description in 1961 (34, 35), TD has been a fascinating lipoprotein disorder. The recent publi-

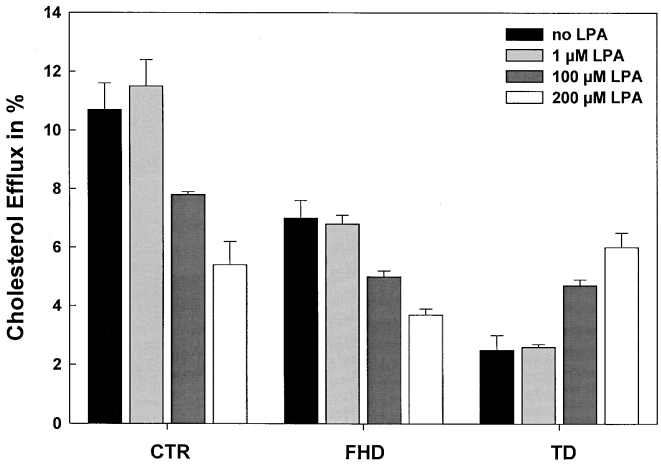

Fig. 8. LPA decreased apoA-I-mediated cellular cholesterol efflux significantly in control and FHD cells, but increased efflux in cells from TD patients.

cation of the genetic defect in TD and familial HDL deficiency (19, 20, 36–40) has shown that FHD represents a heterozygous form of TD. Mutations at the ABCA1 transporter gene have been shown to be associated with TD and FHD. Although the role of ABCA1 is not completely known, it is postulated to act as a transporter of cholesterol and perhaps phospholipids to the plasma membrane, probably from the *trans*-Golgi network.

In the present study, we have shown that apoA-I-mediated cellular cholesterol efflux can be modulated in control, FHD, and TD cells. ApoA-I has been shown to mediate PC-PLC and PC-PLD activation, which leads to increased amounts of DAG, which in turn stimulates PKC (26). We investigated the hypothesis that PKC then activates PLD, resulting in increased amounts of PA, which is necessary for cellular cholesterol efflux. By activating PKC with DOG, PMA, or through the addition of PA to the efflux medium, cellular cholesterol efflux can be increased in a dose-dependent fashion. The potential role of PA is further strengthened by the effect of propranolol, a PAPH inhibitor that increases cellular PA concentration (by inhibiting its conversion to DAG), which increases efflux. As there is no commercially available specific inhibitor of PLD, we used *n*-butanol, which is transphosphatidylated to Pbut by PLD. In the presence of primary alcohols, PC-PLD catalyzes a phosphatidyl transfer reaction, producing phosphatidylalcohols (Pbut, in the presence of *n*-butanol), which are not used as substrates for PAPH. When the cells were incubated simultaneously with DOG and *n*-butanol, efflux was reduced compared with stimulation with DOG only, strongly suggesting that a dual activation of both PLC and PLD occurs after apoA-I stimulation. In addition, PKC can also activate PLD (41), generating PA, which appears to be required for cellular cholesterol efflux. Thus, one likely series of events in the activation of the cholesterol efflux pathway is first the interaction of apoA-I-containing lipoproteins (likely apoA-I-phospholipid discs) with the cell surface and the activation of G-protein-mediated activation of PLC and PLD. The generation of DAG then stimulates PKC, which in turn activates PLD to generate PA. Data from Walter and co-workers (26) reveal that pertussis toxin, a G-protein inhibitor, markedly decreases cholesterol efflux. We have shown in the present study that DOG-stimulated PKC activity could increase cholesterol efflux in the presence of propranolol (which increases PA). Conversely, *n*-butanol (which decreases PA) decreases cellular cholesterol. When this pathway is inhibited by the PKC antagonist calphostin C, a marked reduction in DOG-stimulated cellular cholesterol efflux is observed; however, a lesser degree of inhibition occurred in apoA-I-stimulated cells without DOG. Propranolol can, in part, rescue this inhibition (Fig. 7). We interpret this data as suggesting that apoA-I activates both PC-PLC and PC-PLD.

In contrast to PA, LPA, which is the simplest naturally occurring phospholipid, surprisingly decreased efflux in control and FHD cells, but not in TD cells where an increase in efflux was observed. LPA is known not only as a key intermediate in de novo lipid synthesis, but also as an intracellular phospholipid messenger with a wide range of biological activities, such as stimulation of PLC and PLD, activation of PKC, inhibition of adenylate cyclase, activation of Ras and Rho, and potent Ca^{2+}-mobilizing stimulation (42–47). LPA acts through the activation of a specific G-protein-coupled receptor (28) that can activate multiple LPA-dependent responses (48). In the present study, we cannot rule out an intracellular effect of LPA on cellular cholesterol efflux.

The SM and PC data, as well as the cyclodextrin data (Figs. 2 and 3), reveal that cholesterol efflux onto multilamelar vesicles (formed by PC and SM) or cyclodextrin is identical in control, FHD, and TD cells. This suggests that membrane-associated cholesterol desorbs passively onto phospholipid vesicles and cyclodextrin in a similar fashion in control, FHD, and TD cells. In contrast, apoA-I-mediated cholesterol efflux is enhanced in the presence of phospholipids in all three cell lines, correcting the defect in TD at a SM concentration of 100 μM (please note the Y-axis scale difference between Figs. 2 and 3). The addition of phospholipids into the efflux medium has been shown to increase efflux, most likely by increasing the size of the cholesterol "sink" in the form of vesicles (23). In fibroblasts, approximately 90% of both cholesterol and SM has been reported to be in the plasma membrane (49). This co-localization has been suggested to result from the greater affinity of cholesterol for SM compared with other phospholipids (50, 51) and favors cholesterol efflux (52, 53). It is also possible that SM contributes to the signal transduction pathway through the action of sphingomyelinase, resulting in ceramides and sphingosines.

Hydrophobic amines, such as sphingamine, steryl amine, and imipramine have been shown to inhibit cholesterol transfer from lysosomes to the plasma membrane (53, 54). Sphingosine, also a hydrophobic amine, has been shown to be a potent inhibitor of PKC in cell systems (34). Thus, although it is possible that sphingosine acts by inhibiting PKC activity, its role in increasing cellular translocation to the plasma membrane remains a mechanism to be proven.

Based on these data, we postulate that the ABCA1-mediated cholesterol efflux pathway, shown to be defective in TD and FHD, can either be modulated or bypassed after stimulation by products of PC hydrolysis, DAG, and PA. This raises the possibility that ABCA1 may be a proximal mediator to apoA-I cell interaction in cholesterol efflux or that other pathways of cholesterol movements to the plasma membrane can bypass the ABCA1 pathway. This opens the possibility of increasing the cholesterol efflux cellular pathways for potential therapeutic purposes in ABCA1 defects.

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