Defective Regulation of Phosphatidylcholine-specific Phospholipases C and D in a Kindred with Tangier Disease

Evidence for the Involvement of Phosphatidylcholine Breakdown in HDL-mediated Cholesterol Efflux Mechanisms

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Abstract

The negative correlation between coronary heart disease and plasma levels of HDL has been attributed to the ability of HDL to take up cellular cholesterol. The HDL_3-induced removal of cellular cholesterol was reported to be impaired in fibroblasts from patients with familial HDL deficiency (Tangier disease, TD). In addition, we have recently shown that HDL_3 stimulates the hydrolysis of phosphatidylcholine (PC) in cholesterol-loaded fibroblasts. To investigate whether this cell signaling pathway is involved in cholesterol efflux mechanisms, we compared the HDL_3-induced PC hydrolysis in normal fibroblasts and in fibroblasts from a TD kindred, in whom the HDL_3- and apolipoprotein A-I (apo A-I)–induced mobilization of cellular cholesterol was found to be reduced by 50%. The HDL_3-induced formation of phosphatidic acid (PA) via PC-specific phospholipase D (PC-PLD) was markedly reduced by 60–80% in these cells, whereas the formation of diacylglycerol (DG) via PC-specific phospholipase C (PC-PLC) was two- to threefold enhanced. Defective regulation of PC-PLC and PC-PLD was similarly observed in response to apo A-I and endothelin, but not in response to the receptor-independent stimulation of PC hydrolysis by PMA. A Tangier-like PA and DG formation pattern could be induced in normal cells after preincubation with pertussis toxin, suggesting the involvement of a G-protein. The impaired mobilization of radiolabeled cellular cholesterol in TD cells could completely be overcome by increasing the PA levels in the presence of the PA phosphohydrolase inhibitor propranolol. Conversely, the inhibition of PA formation in the presence of 0.3% butanol as well as the inhibition of DG formation in the presence of the PC-PLC inhibitor D 609 reduced the mobilization of cellular cholesterol both in normal and in TD cells. Our data indicate that the coordinate formation of PA and DG via PC-PLD and PC-PLC is essential for efficient cholesterol efflux. The molecular defect in this TD kindred appears to affect an upstream effector of protein kinase C responsible for the G-protein–dependent regulation of PC-specific phospholipases. (*J. Clin Invest.* 1996. 98:2315–2323.) Key words: lipoproteins, HDL • apolipoprotein A-I • signal transduction • phosphatidylcholines • G-proteins

Introduction

Tangier disease (TD)[1] is a rare HDL deficiency syndrome with autosomal codominant inheritance (1, 2). The disorder is characterized by HDL hypercatabolism (3, 4), the nearly complete absence of HDL particles in the plasma of homozygote patients, and half normal HDL cholesterol levels in heterozygote patients. Moreover, homozygote patients have decreased LDL levels, mild hypertriglyceridemia, and massive cholesterol ester deposition in various tissues. A structural defect in apo A-I, the major protein constituent of HDL, was excluded as the cause of this disease (5). Several abnormalities in cellular phospholipid-, triglyceride-, and cholesterol ester metabolism and the appearance of unusual lysosomes as well as the hyperplasia of the Golgi complex observed in Tangier monocytes and fibroblasts pointed to a defect of cellular lipid metabolism or trafficking (6–8).

The kindred studied in this report was identified in Germany by Assmann et al. (9–18). Its clinical, biochemical, and histopathological manifestations have been described in detail in previous reports. They include the typical quantitative and structural abnormalities of the plasma lipoproteins (9–11), the lipid storage in reticuloendothelial tissues and fibroblasts (10–14), HDL hypercatabolism (15, 16), the erroneous lysosomal degradation of HDL precursors in monocyte-derived macrophages (6), the appearance of unusual lysosomes, hyperplasia of the Golgi complex (8), and the lack of severe atherosclerosis (17). Recently, we could demonstrate that the HDL_3- and the apo A-I–mediated translocation of cellular cholesterol from intracellular pools to the plasma membrane and its efflux into the extracellular medium is reduced by approximately 50% in cultivated fibroblasts from this TD kindred (18). Reduced cholesterol efflux (19, 20) as well as HDL hypercatabolism (3, 4) has similarly been observed in other TD kindreds. It has therefore been speculated that the impaired cellular translocation of cholesterol contributes to the irregular maturation of HDL particles, which then results in hypercatabolism of im-

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1. *Abbreviations used in this paper:* Cho, choline; ChoP, phosphocholine; DG, 1, 2-diacylglycerol; FAFA, fatty acid-free albumin; FC, free cholesterol; G-protein, guanine-nucleotide-binding regulatory protein; MG, monoacylglycerol; PA, phosphatidic acid; PBut, phosphatidylbutanol; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PC-PLD, phosphatidylcholine-specific phospholipase D; PT, pertussis toxin; TD, Tangier disease.

mature HDL precursors (18–20). However, the exact cellular defect is still unclear.

HDL_3 mediates intracellular metabolic responses by binding to specific cell-surface receptors (21), with subsequent breakdown of phospholipids (22). HDL_3 activates both phosphoinositide- and phosphatidylcholine (PC)-hydrolysis. However, under cell culture conditions, the PC-hydrolysis only was influenced by cholesterol loading (22). Thus, the PC breakdown may represent the mechanism promoting cholesterol mobilization. In this paper, we investigated the possibility that the impaired cholesterol translocation and efflux in TD cells is related to defects in cellular PC hydrolysis induced by HDL_3 or apo A-I. PC can be hydrolyzed by PC-specific phospholipase C (PC-PLC) or by PC-specific phospholipase D (PC-PLD). The action of PC-PLC forms diacylglycerol (DG) and phosphocholine (ChoP), PC-PLD produces phosphatidic acid (PA) and choline (Cho). PA and DG can be interconverted to each other by a PA-phosphohydrolase and DG-kinase (Fig. 1). Our data indicate that the coordinate regulation of PC-PLC and PC-PLD is impaired in TD fibroblasts and thus appears to be necessary for regular HDL_3-mediated cholesterol efflux.

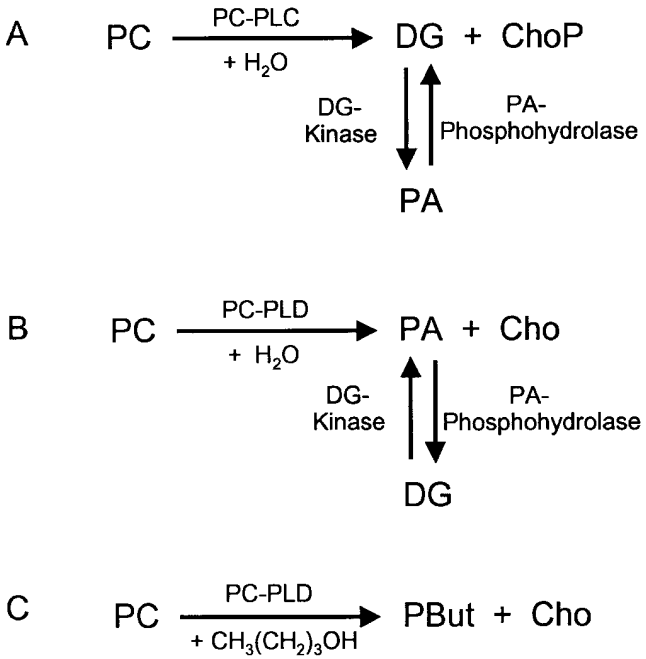

Figure 1. Activities of PC-PLC and PC-PLD. PC-PLC hydrolyzes PC to yield the free polar headgroup ChoP and DG (*A*). PC-PLD hydrolyzes PC to yield Cho and PA (*B*). In the presence of primary alcohols such as butanol PLD catalyzes preferentially a phosphatidyltransfer reaction (*C*), producing phosphatidylalcohols (in the presence of butanol PBut). Because phosphatidylalcohols are not attacked by PA-phosphohydrolase and the production of phosphatidylalcohol is mediated exclusively by PLD, synthesis of phosphatidylalcohols is a specific and unequivocal marker for the involvement of this enzyme.

Methods

Probands. The experiments were performed with fibroblasts from five normolipidemic healthy probands and two Tangier patients of the JS family (patient JS: 62 y, male; patient EG: 59 y, female, sister of JS) whose clinical, biochemical, and histopathological manifestations have been described in detail in several previous reports (6–18).

Materials. L-lyso-3-phosphatidylcholine, [1-^{14}C]palmitoyl (56 mCi/mmol), [methyl-^{14}C]choline chloride (53 mCi/mmol), and [1-^{14}C]arachidonic acid were obtained from Amersham Corp. (Braunschweig, Germany). Autoradiography was performed by using Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Media components were obtained from Flow Laboratories (Meckenheim, Germany). Plastic culture dishes were purchased from Falcon Labware (Oxnard, CA), scintillation-counting mixture (Ultima-Gold) was obtained from Canberra-Packard (Frankfurt, Germany). Aluminium-backed silica gel 60 TLC plates and the solvents for TLC were purchased from Merck (Darmstadt, Germany). The solvents for TLC were of analytical grade. All other reagents were obtained from Sigma Chemical Co. (Deisenhofen, Germany) and were of the highest purity available.

Cell culture. Human skin fibroblasts cultured from biopsies of adult human hip skin were grown and maintained in DME containing 10% FCS, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Once separated, the dermis was cut into small pieces (0.5 mm on each side) and placed in a flask in DME. When these primary cultures were confluent they were expanded by passage. For experiments, cells between passage levels three and six were seeded in 60-mm culture dishes at a density of $\sim 7.5 \times 10^4$/dish. The state of 70–80% confluence was reached after 3–4 d. At the state of 70–80% confluence, the cultures were cholesterol-loaded to upregulate HDL_3 binding (21). Cell layers were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated for 48 h at 37°C in DME supplemented with 50 μg/ml free cholesterol complexed with 10% FCS. In one experiment cells were stimulated with 100 nM PMA (stock solution: 4 mg/ml dissolved in DMSO) or the inactive phorbol ester phorbol 12,13-dibutyrate. In another experiment cells were treated with 100 ng/ml of pertussis toxin from Bordetella pertussis (dissolved in 50% glycerol, 50 mM sodium phosphate, 0.5 M NaCl, pH 7.2) or control buffer (50% glycerol, 50 mM sodium phosphate, 0.5 M NaCl, pH 7.2) for the final 12-h period of cholesterol loading and for the subsequent 3-h incubation period (23). To block PA phosphohydrolase, cells were treated with 100 μmol/liter propranolol for the final 3-h incubation period (24). To inhibit PC-PLC, cells were treated with 30 μg/ml D 609 (10 mg/ml stock solution dissolved in water) for 30 min and for the final 3-h incubation period. Under these experimental conditions, growth and cell viability (as measured by the Trypan exclusion assay) did not differ between Tangier and normal cells, either with or without addition of inhibitors.

Lipoprotein isolation. HDL_3 (d = 1.125–1.210 g/ml) was isolated by standard differential ultracentrifugation from fresh normal human plasma and dialyzed against 0.3 mM Tris-HCl buffer (pH 6.8) containing 0.15 M NaCl (25). Modified HDL_3 (HDL_3-TNM) was prepared as described (26).

Preparation of apo A-I proteoliposomes. Apo A-I proteoliposomes containing normal apo A-I and DMPC were obtained by the cholate dialysis method according to Chen and Albers (27). Apo A-I was prepared from HDL_3 by a procedure described previously (28), lyophilized, and stored as 500-μg aliquots at −70°C. Contamination with other proteins was excluded by analytical isoelectric focusing and subsequent immunoblotting. The molar ratio of apo A-I to DMPC was 1:150. Before incubation with cells, proteoliposomes were dialyzed overnight at room temperature against DME.

Assay for PC-derived radiolabeled PA and DG formation. Fibroblasts were seeded into 60-mm dishes, grown to 70–80% confluence and cholesterol loaded. After 48 h, the medium was removed, the cells were washed three times with PBS and incubated for 2 h in 2 ml of DME-Hepes containing 0.05 μCi/ml L-lyso-3-phosphatidylcholine, [1-^{14}C]palmitoyl (56 mCi/mmol). Thereafter, cells were washed five times in PBS, and incubated for additional 3 h in 2 ml of DME-Hepes. At different times before stopping the 3-h incubation, cells were stimulated with HDL_3, apo A-I proteoliposomes, endothelin 1, PMA, or PBS (control value). To terminate the incubation, the me-

dium was removed and the cell dishes were quickly placed in a liquid nitrogen bath. The cells were scraped from the dishes with a rubber policeman, once with 2 ml of ice-cold methanol and once with 2 ml of ice-cold distilled water. Radioactive lipids were extracted by the method of Folch et al. (29). For transphosphatidylation experiments the 3-h incubation phase was performed in the presence of 0.3% butanol.

Separation of radiolabeled PA, DG, monoacylglycerol (MG), and PBut by TLC. In most experiments, a double one-dimensional TLC (22) was used to separate phospholipids and neutral lipids of interest. In this approach, a series of samples were spotted 12 cm from the bottom of the plate. In order to resolve labeled neutral lipids (DG, MG, fatty acids, and triglycerides) from phospholipids that remained at the origin, the plates were twice developed in toluene/ether/ethanol/triethylamine (100:80:4:2, per vol). After the first run to 20 cm, the plates were thoroughly dried and developed a second time with the same solution to 18 cm. Plates were then cut 0.8 cm above the origin (i.e., 12.8 cm above the lower edge of the plate), rotated 180°, and developed to the top with chloroform/methanol/ammonium hydroxide (65:35:5 per vol). After they were dried thoroughly, autoradiography was performed by using Kodak X-OMAT film for 7–14 d. Radioactive bands were cut from the silica plates, placed in scintillation vials containing 10 ml Ultima-Gold scintillation fluid, and quantitated by liquid scintillation counting in a scintillation counter (model 1214; LKB, Bromma, Sweden). The identities of labeled bands were determined based on R_F values obtained for authentic neutral lipids and phospholipids visualized by iodine staining. Phosphatidylbutanol (PBut) standard was prepared as described in Kaszkin et al. (30).

Measurement of choline release. Fibroblasts were labeled for 48 h in choline-free DME containing [*methyl*-^{14}C]choline (2 μCi/ml), 10% FCS, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution. During the labeling interval 30–40% of the [^{14}C]choline was taken up by the cells. After 48 h the medium was removed, the cells were washed three times with PBS and incubated for 20 min in 2 ml of DME-Hepes containing unlabeled choline (1 mM) for 20 min. Cells were washed in PBS, and incubated for further 20 min in fresh choline-free DME-Hepes. The medium was removed, the cells washed again twice in 1 ml of PBS, and incubated in 1 ml of choline-free DME-Hepes for additional 3 h. At different times before stopping the 3-h incubation, cells were stimulated with either HDL_3 (20 μg/ml) or PBS. After removing the medium, the cell dishes were rapidly immersed in a liquid nitrogen bath and stored at −20°C until further preparation. The cells were scraped off the dishes using a rubber policeman, once with 2 ml of ice-cold methanol, and once with 2 ml of ice-cold distilled water. The aqueous and lipid phases of the cell extracts were separated as described by Folch et al. (29). The aqueous phase of the chloroform/methanol extract was dried and resuspended in 50% ethanol.

Separation of [^{14}C]choline-labeled metabolites. Aqueous [^{14}C]choline-labeled metabolites were separated and quantitated by a procedure modified from that described by Yavin et al. (31). Nonradioactive choline and phosphocholine (50 μg of each) were added to an aliquot of the aqueous phase, which amounted to one-half of the total. The samples with standards added were dried by vacuum centrifugation. The residue was dissolved in 75 μl of 50% ethanol, spotted on to silica gel 60 plates, and developed to the top in a solvent composed of 0.6% NaCl/methanol/35% NH_3 (100:100:3, per vol). The plates were air dried for 30 min, and the standards were visualized by exposure of the plates to iodine vapor. Spots corresponding to Cho (R_F 0.09) and ChoP (R_F 0.39) were cut out, eluted for 48 h with 10 ml of Ultima-Gold scintillation fluid, and then counted for radioactivity by liquid scintillation in a scintillation counter (model 1214; LKB, Bromma, Sweden).

Mobilization of cellular cholesterol. Intracellular sterols were labeled by pulse incubations with [^{14}C]mevalonolactone as previously described (18). After incubation in cholesterol-containing medium, cell layers were rinsed three times with PBS containing 1 mg/ml fatty acid-free albumin (FAFA), and were then incubated for 4 h at 37°C in bicarbonate-free DME-Hepes, 1 mg/ml FAFA, 8 μCi/ml [^{14}C]mevalonolactone, 0.5 mM mevalonolactone, and 2 μg/ml of the ACAT inhibitor octimibate (Rhône Poulenc). After incubation, cell layers were rinsed three times with PBS-FAFA, and then incubated in the same media without label for 1 h at 37°C. Cell layers were rinsed once more in PBS-FAFA and then incubated for 2 h at 37°C in bicarbonate-free DME-Hepes, 1 mg/ml FAFA, in the presence of HDL_3 or PBS (basal value). After a 2-h incubation period in absence or presence of HDL_3, the efflux media were collected, 1 ml of PBS-FAFA was used to rinse the cell layers, and the wash was added to the efflux media. Efflux media were extracted three times by the method of Folch et al. (29). Sterol species were separated by thin-layer chromatography on silica gel G plates (Merck, Darmstadt, Germany) developed in heptane/ether/methanol/acetic acid (80:30:10:1.2, per vol). Lipids were identified by staining with I_2 vapor and by comigration with standards. Appropriate spots were taken for scintillation counting. For the determination of specific activities, the cellular cholesterol mass was determined by a fluoroenzymatic method (32).

General procedures. Protein concentrations were measured according to the method of Bradford et al. (33), using bovine serum albumin as the standard. Unless otherwise indicated, each experiment was performed in triplicate and repeated three to five times. Data represent the mean±SD of data obtained from a representative experiment. Student's *t* test was used for the comparison of mean values.

Results

HDL_3-induced activation of PC-PLD and PC-PLC in normal and TD fibroblasts. In cholesterol-loaded human skin fibroblasts from normolipidemic probands, HDL_3 activates PC-PLD in a biphasic manner (Fig. 2 *A*). Both products of PC-PLD, PA, and Cho, increased with two peak maxima at 30 to 60 s and 7 to 10 min. Activation of PC-PLD was confirmed by the accumulation of phosphatidylbutanol (PBut) in the presence of butanol (Fig. 2 *A*). In the presence of primary alcohols PC-PLD catalyzes a phosphatidyl-transfer reaction, producing phosphatidylalcohols (in the presence of butanol PBut), which are not attacked by PA-phosphohydrolase. Because the production of phosphatidylalcohols is mediated exclusively by PLD (Fig. 1), the accumulation of PBut is a specific marker for the involvement of this enzyme (34, 35). In Tangier fibroblasts, the HDL_3-mediated increase of PA and Cho occurred with a lag phase of 5–10 min, and the maximal increase was reduced by 70–80% (Fig. 2 *B*), indicating a reduced activity of PC-PLD. The biphasic kinetics of PA and Cho formation seen in control cells was barely recognizable. In addition, PC-PLD-mediated transphosphatidylation in response to HDL_3 was reduced by 60–80%, confirming impaired activation of PC-PLD (Fig. 2 *B*).

The direct products of PC-specific PLC (PC-PLC) are DG and ChoP (Fig. 1). In normal fibroblasts, a rapid increase of DG and a small transient rise of ChoP was observed 2–5 min after stimulation, followed by a slow gradual increase of DG and ChoP for at least 2 h (Fig. 2 *C*). Because DG can also be converted from PA by PA phosphohydrolase, we estimated the true portion of PC-PLC–mediated DG formation, by comparing the increases of PC-derived DG in the absence and presence of butanol, which traps PLD-derived PA as PBut. As shown in Table I, the major part of DG at 2, 5, and 10 min after stimulation was inhibited in the presence of 0.3% butanol in normal fibroblasts, suggesting a major precursor role for PA in HDL_3-induced DG formation. Conversely, at longer incubation times (30 min, 60 min) the DG response was inhibited to a lesser degree, indicating increasing influence of PC-PLC on

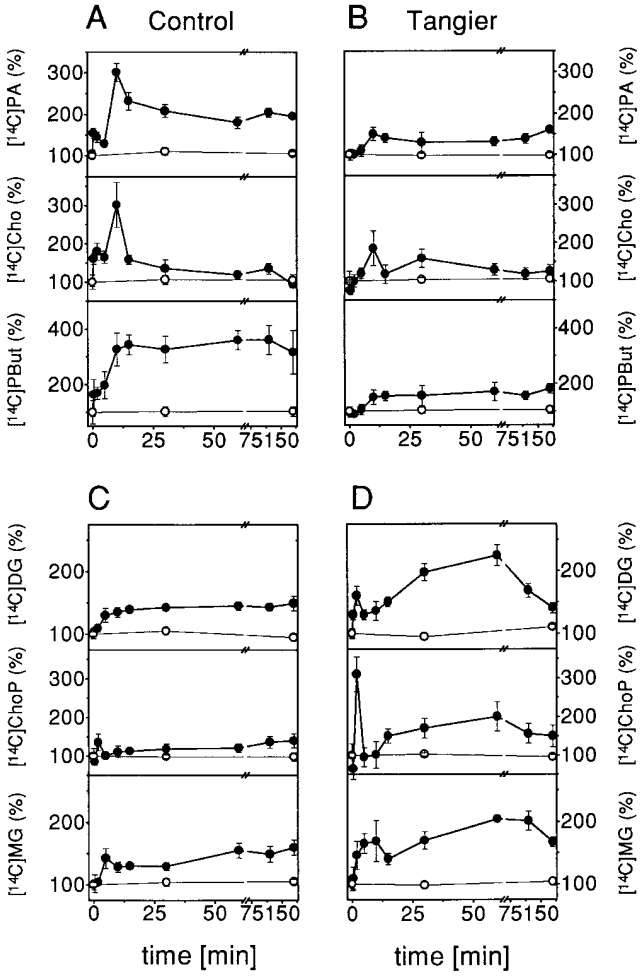

Figure 2. HDL_3-Induced activation of PC-PLD and PC-PLC in normal and TD fibroblasts. The products of PC-PLD (PA, PBut, and Cho) are presented for normal (*A*) and TD fibroblasts (*B*), and the products of PC-PLC (DG, ChoP) and MG are presented for normal (*C*) and TD fibroblasts (*D*), respectively. Cholesterol-loaded human skin fibroblasts were prelabeled with [^{14}C]lyso-PC (measurement of PA, PBut, DG, and MG) or with [^{14}C-methyl]choline chloride (measurement of Cho and ChoP), as described in Methods. At the indicated times 20 μg/ml HDL_3 (*closed symbols*) or PBS buffer (*open symbols*) was added to control fibroblasts (*A*, *C*) or TD fibroblasts (*B*, *D*). The amount of [^{14}C]radioactivity in PA, DG, PBut, and MG (in dpm) was normalized for total radioactivity separated on TLC, and was then expressed as the percentage of the mean for the basal value (incubation without HDL). In the presented experiment, the total amount of radioactivity incorporated into cellular lipids and separated on TLC was 33,476±9,256 dpm in normal and 42,673±5,498 dpm in TD cells. The basal dpm values for PA, DG, PBut, and MG ranged between 800 and 1100, 400 and 600, 300 and 500, 100 and 250 dpm, respectively. These were not significantly different in normal and TD cells. Values for Cho and ChoP were expressed as the percentage of the mean for the basal value (incubation without HDL). Mean basal values were: 10,478±1,987 dpm (Cho, control), 34,589±6,786 dpm (ChoP, control), 9,678±2,467 dpm (Cho, TD), and 44,529±11,983 dpm (ChoP, TD). Values are the mean±SD for three determinations from a typical experiment out of five. Comparable results were obtained on five controls and two homozygous Tangier patients.

DG formation. That cellular ChoP accumulates at longer incubation times (Fig. 2 *C*) is consistent with an increasing activation of PC-PLC. The formation of DG was paralleled by the formation of MG, the relative increase of which was comparable to or even slightly higher than that of DG. MG was most likely derived from DG by the action of DG lipase (36). Triacylglycerol levels were not significantly influenced by HDL_3 (not shown).

Despite the reduced PLD activity, the increases in the levels of PC-derived DG and MG were two- to threefold more noticeable in Tangier cells than in controls (Fig. 2 *D*). In addition, only a minor portion of DG formation at 2, 5, and 10 min after stimulation was inhibited by butanol (Table I). This indicated that normal and Tangier cells activate different pathways of PC breakdown. DG in Tangier cells was mainly derived directly from PC by PC-PLC, whereas in normal cells DG was mainly produced by the consecutive action of PC-PLD and PA-phosphohydrolase.

PC breakdown in response to apo A-I, endothelin and PMA in normal and TD fibroblasts. The putative HDL_3 receptor is a 105-kD protein, which is upregulated in cholesterol-loaded cells (37). Apo A-I, the main protein constituent of HDL_3, is the likely physiological ligand for this receptor (38). We recently demonstrated that only the early activation of PC-PLD (resulting in the first PA peak in Fig. 2 *A*) can be mimicked by apo A-I, and that only this second messenger response is inducible by cholesterol loading of the cells (22). The late PC-hydrolysis may be induced by another as yet unidentified agonist on the HDL particle. The results shown in Fig. 2 appear to indicate that enhanced activation of PC-PLC and diminished activation of PC-PLD in TD fibroblasts includes both phases of HDL_3-induced PC-hydrolysis. To examine the apo A-I–induced PC-breakdown in more detail, apo A-I proteoliposomes (10 μg/ml) were tested for their ability to induce PC hydrolysis in normal and Tangier fibroblasts. The kinetics and the extent of PA and DG increases induced by apo A-I proteoliposomes closely resembled the first phase observed with 20 μg/ml HDL_3. As shown in Fig. 3, *A* and *B*, the PA response in control cells preceded the DG increase, indicating that DG formation occurred at least partly by a coupled PLD/PA-phosphohydrolase pathway. This suggestion was further strengthened by the observation that the apo A-I–induced DG formation was decreased in the presence of 0.3% butanol (Table I). Activation of PC-PLD was confirmed by transphosphatidylation in the presence of 0.3% butanol (Table II). In Tangier fibroblasts, by contrast, activation of PC-PLD was markedly reduced and delayed. Despite the inhibition of PC-PLD, the PC-derived DG increase was not found to be decreased but was rather slightly increased. In contrast to normal cells, the DG formation preceded PA formation in TD fibroblasts (Fig. 3, *A* and *B*), and was not influenced by butanol (Table I). These results pointed to uncoordinated activation of PC-PLC and PC-PLD in TD cells. In order to examine whether this is also demonstrable with an agonist that does not bind to the HDL receptor, we used endothelin 1 as agonist. This vasoconstrictor binds to specific endothelin receptors on fibroblasts, and stimulates PC hydrolysis through both PLC and PLD pathways (39). As demonstrated by the accumulation of PBut in the presence of 0.3% butanol (Table II), 10 nM endothelin induced activation of PC-PLD in normal cells. The rapid conversion of PA to DG apparently prevented the accumulation of PA and resulted in a transient decrease of basal PA levels

Table I. Influence of Butanol on the HDL_3-Induced [^{14}C]DG Formation

	Radioactivity (dpm/dish)			
	Normal		Tangier	
	Butanol −	Butanol +	Butanol −	Butanol +
No addition	640±50 (100)	720±70 (100)	730±89 (100)	820±62 (100)
20 μg/ml HDL_3, 1 min	655±23 (103)	715±76 (99)	956±93 (131)	1128±74 (138)
20 μg/ml HDL_3, 2 min	779±46 (122)	749±39 (104)	1350±112 (185)	1314±96 (160)
20 μg/ml HDL_3, 5 min	930±38 (145)	799±66 (111)*	1190±130 (163)	1188±84 (145)
20 μg/ml HDL_3, 10 min	1020±64 (159)	857±52 (119)*	1242±154 (170)	1362±64 (166)
20 μg/ml HDL_3, 30 min	1066±122 (166)	986±99 (137)	1416±66 (194)	1254±54 (153)*
20 μg/ml HDL_3, 60 min	1042±24 (163)	1030±114 (143)	1529±108 (209)	1368±95 (167)
10 μg/ml apo A-I, 1 min	966±40 (151)	842±55 (117)*	1132±63 (155)	1190±86 (145)
10 nM endothelin, 2 min	1792±84 (280)	1148±135 (135)‡	1898±84 (260)	1804±65 (220)

Normal and Tangier fibroblasts were cholesterol-loaded and radiolabeled with [^{14}C]lyso-PC as described for Fig. 2. The cells were then incubated for 3 h in DME-Hepes in absence or presence of 0.3% butanol. At the indicated times before terminating the incubation period, 20 μg/ml HDL_3, 10 μg/ml apo A-I proteoliposomes or 10 nM endothelin was added. Lipid extraction and determination of radioactivity in DG and PA were performed as described in Methods. The amount of [^{14}C] radioactivity in DG was expressed as dpm/dish. Each value is the mean±SD of triplicate incubations. Values in parentheses are stimulations expressed as a percentage of the control value (no addition). The experiment is representative for three experiments from fibroblasts of two homozygous TD patients and three control subjects. *$P < 0.05$, ‡$P < 0.01$ comparing by Student's *t* test the mean values and standard deviations for [^{14}C]DG formation in presence of butanol to the respective values in absence of butanol.

(Fig. 3 *C*), accompanied by DG increases up to 300–350% of basal values, 2 min after stimulation (Fig. 3 *D*). In TD fibroblasts, PC-PLD activation in response to 10 nM endothelin was reduced and occurred with a lag phase (Fig. 3 *C*; Table II). However, the initial DG increase was higher than in control cells, reaching statistical significance at 30 s ($P < 0.001$, Student's *t* test), when DG in normal cells was not significantly increased (Fig. 3 *D*). In contrast to the controls, the stimulation of DG formation was only slightly affected in the presence of butanol (Table I), suggesting that endothelin-stimulated DG accumulation in TD fibroblasts was a consequence of PC-PLC activation.

To further examine whether the defective regulation of PC-PLC and PC-PLD observed in TD fibroblasts is ligand-dependent, PC hydrolysis was stimulated with PMA, an activator of protein kinase C (PKC), that activates phospholipases directly (40). As shown in Fig. 3 *E* and *F*, the PMA-stimulated PC-hydrolysis was not different in normal and TD cells. In contrast to HDL_3, apo A-I and endothelin, PMA activated PC-PLD subsequent to PC-PLC, indicated by the observation that both PA and PBut formation followed the increase of DG (Fig. 4 *E* and *F*; Table II).

HDL_3-induced PC breakdown in PT-treated normal and TD fibroblasts. The above presented data indicated that the regulation of PC-PLC and PC-PLD is impaired in Tangier cells. The defect is apparently neither agonist- nor receptor-specific. In this context, it is interesting to note that phospholipases are coupled to cell surface receptors by stimulatory or inhibitory guanine nucleotide-binding regulatory proteins (G-proteins), which may interact with various receptors (41). In rat fibroblasts, PC-PLD was found to be regulated by a PT-sensitive stimulatory G-protein (42). To examine the G-protein coupled processes in response to HDL_3 in more detail, we investigated the ability of pertussis-toxin (PT), which catalyzes ADP-ribosylation of G-proteins, to block phospholipase activation. In cells treated with 100 ng/ml PT per ml for 12 h, PT-sensitive GTP-binding proteins are completely inhibited by PT-catalyzed ADP-ribosylation of their α-subunits (23). As shown in Fig. 4, PT-pretreatment of normal and TD fibroblasts completely blocked HDL_3-induced activation of PC-PLD. Increases of DG, by contrast, were markedly enhanced in normal, and slightly enhanced in TD cells. These data could best be explained by an inverse coupling of PC-PLD and PC-PLC by a G-protein dependent process. It is evident from Fig. 4 that PT treatment of normal fibroblasts resulted in a PA and DG formation pattern very similar to that observed in non-PT–

Table II. Endothelin-, Apo A-I-, and PMA-Induced [^{14}C]PBut Formation

	Radioactivity (dpm/dish)	
Treatment	Normal	Tangier
No addition	121±21 (100)	101±21 (100)
10 μg/ml apo A-I, 2 min	211±36 (174)*	124±23 (123)‡
10 nM endothelin, 2 min	260±43 (215)§	142±43 (142)‡
100 nM PMA, 5 min	164±33 (136)	139±37 (138)
100 nM PMA, 10 min	210±55 (174)*	187±47 (185)*

Normal and Tangier fibroblasts were cholesterol-loaded and radiolabeled with [^{14}C]lyso-PC as described for Fig. 2. The cells were then incubated for 3 h in DME-Hepes in absence or presence of 0.3% butanol. 2, 5, or 10 min before terminating the 3 h incubation period, 10 μg/ml apo A-I proteoliposomes, 10 nM endothelin, or 100 nM PMA was added. Each value is the mean±SD of triplicate incubations. Values in paretheses are stimulations expressed as a percentage of the basal value (no additions). The experiment is representative of three experiments from fibroblasts of two homozygous TD patients and three control subjects. *$P < 0.05$, ‡$P < 0.05$ by Student's *t* test for normal vs. Tangier, and §$P < 0.01$ comparing by Student's *t* test the mean values and standard deviations for [^{14}C]PBut formation in response to apo A-I proteoliposomes, endothelin, and PMA to the basal [^{14}C]PBut formation (no addition).

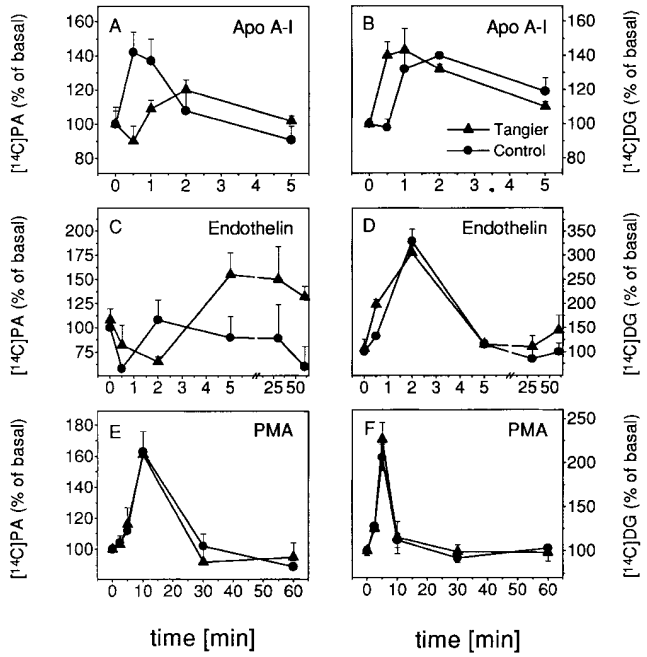

Figure 3. PC breakdown in response to apo A-I, endothelin, and PMA in normal and TD fibroblasts. The figure shows the time courses of [^{14}C]PA (*A*, *C*, *E*) and [^{14}C]DG (*B*, *D*, *F*) formation in control fibroblasts (*circles*) and TD fibroblasts (*triangles*) prelabeled with [^{14}C]lyso-PC and stimulated with apo A-I proteoliposomes, endothelin, or PMA. Control or TD cells were prelabeled with [^{14}C]lyso-PC, as described in the Methods section. At the indicated times 10 μg/ml apo A-I proteoliposomes (*A*, *B*), 10 nM endothelin (*C*, *D*), or 100 nM PMA (*E*, *F*) was added. Values are the mean±SD for three determinations from a typical experiment out of three. The amount of [^{14}C]radioactivity was expressed as dpm. All values were normalized for total radioactivity separated on TLC, and were then expressed as the percentage of the mean for the basal value (incubation without agonist). In the presented experiment, the total amount of radioactivity incorporated into cellular lipids and separated on TLC was 44,639±3,562 dpm in normal and 52,411±7,391 dpm in TD cells. The basal dpm values for PA and DG ranged between 900 and 1,300, and 550 and 650 dpm, respectively. They were not significantly different in normal and TD cells. Each value represents the mean±SD for three determinations from a typical experiment out of five (with fibroblasts from two different TD patients and three different controls).

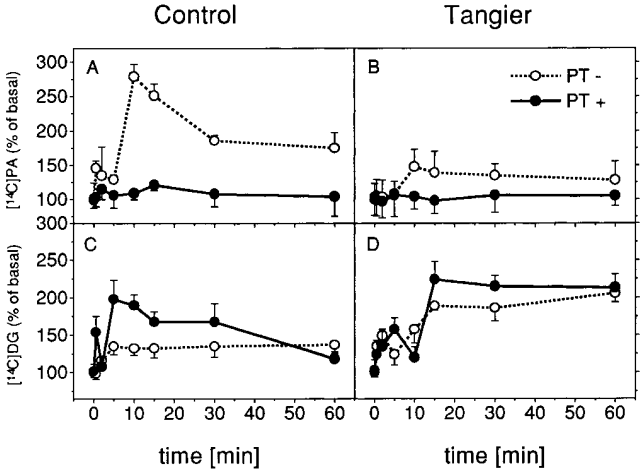

Figure 4. HDL_3-induced PC breakdown in pertussis toxin treated normal and TD fibroblasts. Normal (*A*, *C*) and TD (*B*, *D*) cells were treated and stimulated, as described in Fig. 2, but for the final 12-h period of cholesterol loading cells were treated with 100 ng/ml of pertussis toxin from Bordetella pertussis (*closed symbols*) or control buffer (*open symbols*). PA (*A*, *B*) and DG (*C*, *D*) was measured as described in Fig. 2. Each value was expressed as the percentage of the mean for the basal value (incubation without HDL_3), and represents the mean for three determinations from a typical experiment out of five. The total amount of radioactivity incorporated into cellular lipids and separated on TLC was 30,361±1,492 dpm in normal and 33,945±3,875 dpm in TD cells. The basal dpm values for PA and DG ranged between 650 and 950 and 300 and 600 dpm, respectively.

treated Tangier cells. However, the HDL_3-induced PA formation in TD cells was not completely inhibited, and the enhanced DG levels in response to HDL_3 were still enhanced by PT pretreatment in five of six independent experiments (Fig. 4). These data suggest that under physiological conditions (without PT) PC-PLD is stimulated via a G-protein coupled process, and that PC-PLC is simultaneously inhibited. This mechanism is apparently distorted, but not totally defective in cells from this TD kindred.

Influence of propranolol, butanol, D 609, and PT on the mobilization of cellular cholesterol in normal and Tangier fibroblasts. The ability of HDL_3 to promote efflux of cellular free cholesterol (FC) was compared in normal and TD fibroblasts in the absence or presence of substances that influence PC-PLC or PC-PLD. Cholesterol-loaded cells were treated with 2 μg/ml of the ACAT inhibitor octimibate to prevent esterification of de novo synthesized cholesterol. The cells were then pulse-labeled with [^{14}C]mevalonolactone to enrich intracellular pools with labeled sterol. After an additional 2-h incubation period in the presence of 20 μg/ml HDL_3 and 1 mg/ml FAFA, the [^{14}C]FC in the extracellular medium was quantified as described in the Methods section. The values obtained with FAFA but without HDL_3 were set as 100% (basal value). Under basal conditions, neither the total amount of radioactivity incorporated into cellular FC (control: 62,766±5,478 dpm/mg cell protein; Tangier: 59,765±4,237 dpm/mg cell protein) nor the specific activities of cellular FC (control: 425±63 dpm/μg FC; Tangier: 495±47 dpm/μg FC) were significantly different in the two cell types.

As shown in Fig. 5, the addition of 20 μg/ml HDL_3 to control cell cultures caused an increase of extracellular [^{14}C]FC up to 385±32% of the basal value during the 2-h incubation period. In TD fibroblasts, the addition of 20 μg/ml HDL_3 to the cell cultures caused an increase of extracellular [^{14}C]FC up to 177±19% of the basal value. Thus, the mobilization of cellular cholesterol was markedly decreased by 54%, compared to controls ($P < 0.001$, Student's *t* test).

In the presence of propranolol (100 μM), which inhibits PA phosphohydrolase with a resulting increase in the levels of PA (24), the HDL_3-induced efflux of cellular cholesterol was enhanced by 19% in normal fibroblasts (458±43% vs. 385±32% of the basal value, $P < 0.05$, Student's *t* test) and by 97% to almost normal basal values in Tangier fibroblasts (349±23% vs. 177±19% of basal, $P < 0.001$, Student's *t* test). PA levels were increased in parallel by 21% in normal cells ($P < 0.01$, Student's *t* test, Table III) and by 27% in TD cells ($P < 0.01$, Student's *t* test, Table III). DG levels in the presence of propranolol were significantly decreased in normal ($P < 0.05$, Student's *t* test, Table III), but not in TD cells.

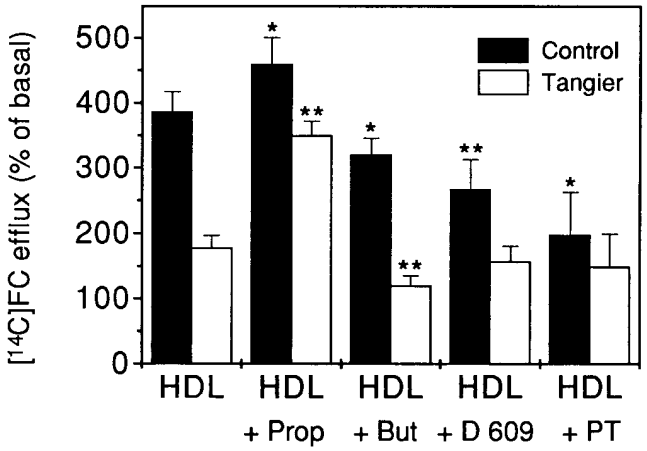

Figure 5. Influence of propranolol, butanol, D 609, and pertussis toxin on the mobilization of cellular cholesterol in normal and TD fibroblasts. Cholesterol-loaded normal and TD fibroblasts were pulse-labeled with [^{14}C]mevalonolactone (8 μCi/ml, 0.5 mM). After incubation for 4 h at 37°C in bicarbonate-free DME-Hepes, 1 mg/ml FAFA, 8 μCi/ml [^{14}C]mevalonolactone, 0.5 mM mevalonolactone, and 2 μg/ml of the ACAT inhibitor octimibate, cell layers were rinsed three times with PBS-FAFA, and then incubated in the same media without label for 1 h at 37°C. Cell layers were rinsed once more in PBS-FAFA and then incubated for 2 h at 37°C in bicarbonate-free DME-Hepes, 1 mg/ml FAFA, in the presence or absence of HDL_3 (20 μg/ml). [^{14}C]FC in the efflux medium was extracted and separated as described in Methods, and was expressed as the percentage of the mean for the basal value (incubation in the presence of FAFA alone). To block PA phosphohydrolase, cells were treated with 100 μmol/l propranolol for the 2-h incubation period. To inhibit PC-PLC, cells were treated with 30 μg/ml D 609 for 30 min and for the 2-h incubation period. 0.3% butanol was added for the 2-h incubation period. 100 ng/ml of pertussis toxin from Bordetella pertussis were added for the final 12-h period of cholesterol loading and for the subsequent incubation period. $*P < 0.05$, $**P < 0.01$; Student's *t* test comparing pretreatment and no pretreatment (HDL alone); ($n = 4$).

In the presence of 0.3% butanol, which traps PA such as PBut, the cholesterol efflux was inhibited by 17% in normal cells (320±26% vs. 385±32% of basal, $P < 0.05$, Student's *t* test) and by 33% in TD fibroblasts (119±16% vs. 177±19% of basal, $P < 0.01$, Student's *t* test). PA levels were concomitantly decreased by 13% in normal fibroblasts ($P < 0.05$, Student's *t* test, Table III) and by 21% in TD fibroblasts ($P < 0.05$, Student's *t* test, Table III).

In the presence of 30 μM D 609 (a specific PC-PLC inhibitor), the cholesterol efflux was inhibited by 31% in normal cells (266±47% vs. 385±32% of basal, $P < 0.05$, Student's *t* test), and by 12% in TD cells (156±24% vs. 177±19% of basal, not significant). PC-derived DG levels were decreased by 17% in normal cells ($P < 0.01$, Student's *t* test, Table III) and by 22% in TD cells by this treatment ($P < 0.01$, Student's *t* test, Table III). PA levels were not significantly influenced by D 609.

Pretreatment of the fibroblasts with PT decreased the HDL_3-induced cholesterol efflux by 40% in control fibroblasts (231±67% vs. 385±32% of basal, $P < 0.01$, Student's *t* test) and by 16% in TD fibroblasts (148±51% vs. 177±19% of basal, not significant, Student's *t* test). DG levels were enhanced, and PA levels were suppressed to almost basal values in the presence of PT (Table III).

In summary, both inhibition of PC-PLC and inhibition of PC-PLD was associated with reduced mobilization of cellular cholesterol. In TD cells, the inhibition of PA formation reduced cholesterol efflux to a greater extent than in normal cells, whereas the inhibition of DG formation reduced cholesterol efflux to a lesser extent than in controls. Conversely, the pharmacological increase of PA enhanced cholesterol efflux to almost normal levels in TD cells, but only slightly increased cholesterol efflux in control cells. PT reduced cholesterol efflux in normal, but not in TD fibroblasts.

Table III. Effect of Propranolol, Butanol, D 609, and Pertussis Toxin on the HDL_3-induced [^{14}C]DG and [^{14}C]PA Formation

	Radioactivity (% of basal)			
	Normal		Tangier	
Treatment	DG	PA	DG	PA
HDL_3	139±5	143±3	176±10	112±7
HDL_3 + Propranolol	117±7*	173±7‡	180±14	142±5‡
HDL_3 + Butanol	119±12*	125±9*	169±12	89±13*
HDL_3 + D 609	115±4‡	147±4	137±7‡	107±6
HDL_3 + Pertussis toxin	172±8‡	106±9‡	201±8*	99±11

Normal and Tangier fibroblasts were cholesterol-loaded and radiolabeled with [^{14}C]lyso-PC as described in Fig. 2. The cells were then incubated for 3 h in DME-Hepes in absence or presence of an inhibitor. 4 min before terminating the 3-h incubation period, 20 μg/ml HDL_3 was added. Lipid extraction and determination of radioactivity in DG and PA were performed as described in Methods. All values were normalized for total radioactivity incorporated into cellular lipids (30,000–60,000 dpm per data point), and were then expressed as the percentage of the mean for the basal value (no addition or addition of the respective inhibitor alone). The treatment with 100 μmol/l propranolol, 0.3% butanol, or 30 μg/ml D 609 was performed during the final 3-h incubation period. 100 ng/ml of pertussis toxin from Bordetella pertussis were added for the final 12-h period of cholesterol loading and for the 3-h incubation period. Each value is the mean±SD of triplicate incubations. The experiment is representative of three experiments from fibroblasts of two homozygous TD patients and three control subjects. $*P < 0.05$; $‡P < 0.01$ comparing by Student's *t* test HDL_3 and HDL_3 + inhibitor ($n = 4$).

Discussion

In this investigation, we demonstrate that the PT-sensitive regulation of PC-PLD and PC-PLC is distorted in TD fibroblasts. The opposite kinetics for the release of the lipid-soluble products of PC-PLC (DG) and PC-PLD (PA) in control and TD fibroblasts was the first indication for abnormal activation of PC-PLC and PC-PLD. However, PA and DG can be interconverted by hydrolases and kinases (Fig. 1). It was therefore necessary to estimate the portion of PLC-mediated DG formation by comparing the increases of PC-derived DG in the absence or presence of butanol, which traps PA such as PBut. In addition, we monitored the formation of PBut as marker of PLD activation. Moreover, the direct water-soluble products of PC-PLC (ChoP) and PC-PLD (Cho) were measured. Using these techniques we demonstrate that the maximum activation of PC-PLD in response to HDL_3 is reduced by 70–80% in TD fibroblasts, and that it is delayed by 5–10 min. Activation of PC-PLC, by contrast, has an early onset, and is two- to threefold enhanced.

The abnormal activation of PC-PLD and PC-PLC was observed in response to HDL_3, apo A-I, and endothelin, but not in response to PMA. The putative HDL_3 receptor is a 105-kD protein, that is upregulated in cholesterol-loaded cells (37). Endothelin, by contrast, is a strong vasoconstrictor, that binds to specific endothelin receptors on fibroblasts (39). Conversely, PMA activates protein kinase C, which in turn stimulates PC-PLC and PC-PLD directly (40). These data indicate that the impaired regulation of PC-PLC and PC-PLD in TD fibroblasts is not receptor or agonist specific. In addition, the experiments with PMA suggest that the phospholipases themselves are not defective, and that the molecular defect is located upstream of PKC. In rat fibroblasts, PC-PLD was found to be regulated by a PT-sensitive stimulatory G-protein (42). We therefore examined the influence of PT on HDL_3-induced PC-hydrolysis. Pretreatment of normal fibroblasts with PT completely blocked activation of PC-PLD, and activation of PC-PLC was markedly enhanced by this treatment. These data indicate the under normal conditions (without PT) the G-protein-dependent activation of PC-PLD is associated with the inhibition of PC-PLC. An inverse coupling of PC-PLC and PC-PLD has previously been described in fibroblasts (43). Moreover, this result is of interest insofar as a DG and PA formation pattern similar to that observed in Tangier cells can be induced in normal fibroblasts by pretreatment of the cells with PT. However, the reduced PA formation in response to HDL_3 in TD cells was further deepened, and the enhanced DG levels in response to HDL_3 were still enhanced in PT-pretreated cells. Thus, a heterotrimeric G-protein or another factor involved in the G-protein-dependent regulation of cellular phospholipases appears to be distorted but not totally defective in fibroblasts from the TD kindred described here. Alternatively, an inadequate supply of phospholipase substrates may occur in these cells. In this context, it is worth notice that abnormal plasma membrane phospholipid content was noted in TD (44). Moreover, dysregulation of phospholipid synthesis as well as diminished phosphoinositide breakdown were found in TD fibroblasts (7, 45). Hence, the possibility that a protein involved in cellular phospholipid transport and distribution is defective in TD can also not entirely be excluded.

Recent investigations demonstrated an impaired HDL_3- and apo A-I–induced mobilization of cellular cholesterol in TD fibroblasts (18–20). On the other hand, we have shown that HDL_3 activates both PI- and PC-turnover (22). However, only the liberation of PC-derived PA and DG was inducible by both HDL_3 and apo A-I, and only this second messenger response was sensitive to cholesterol loading of the cells (22). It was therefore of interest whether there is a causal relationship between cholesterol efflux and HDL_3- and apo A-I–induced PC breakdown. The data described here support the notion that the PC-derived second messengers DG and PA are directly involved in the HDL_3- and apo A-I–induced cholesterol efflux. The impaired HDL_3- and apo A-I–induced mobilization of cellular cholesterol in TD fibroblasts occurred in parallel with the distorted liberation of PC-derived DG and PA. In addition, a Tangier-like PA and DG formation pattern as well as reduced cholesterol efflux was observed in normal PT-treated cells. Moreover, the inhibition of PC-PLC or PC-PLD in the presence of D 609 or butanol, respectively, significantly inhibited the mobilization of cellular cholesterol. Conversely, the pharmacological increase of PA in the presence of propranolol, which inhibits PA phosphohydrolase, enhanced cholesterol efflux to almost normal values in TD cells. Interestingly, the cell type with reduced PC-PLD and enhanced PC-PLC activity (TD) was more sensitive to pharmacological modulation of PC-PLD and less sensitive to modulation of PC-PLC activity. These data suggest that the coordinate formation of PA and DG via PC-PLD and PC-PLC is required for normal cholesterol efflux.

Mendez et al. have shown that the HDL_3-receptor-mediated translocation of cellular cholesterol is dependent on activation of PKC (46). DG is the physiological activator of PKC. Our data indicate that DG formation per se is not sufficient for cholesterol efflux. Cholesterol efflux was impaired in TD fibroblasts despite the fact that PC-derived DG formation was two- to threefold enhanced in response to HDL_3. It has previously been suggested that PA not only functions as a precursor of DG, but can itself also induce important second messenger functions, e.g., in mitogenesis or secretion (47). It has been proposed that PA can transiently alter membrane structures by biophysical means (48). It is therefore conceivable that PA may act as a second messenger involved in cellular cholesterol translocation processes.

In view of the strong inverse relationship between HDL cholesterol levels and coronary risk (49), lack of severe atherosclerosis in the TD kindred described here is remarkable (17). The findings show that the nearly completely absence of HDL, accompanied by impaired cellular cholesterol homeostasis and foam cell formation, is not necessarily associated with the development of atherosclerosis. Several reasons may account for this phenomenon. The cholesterol efflux in response to HDL_3 is not completely absent in the patients described here. Other TD patients have been reported with a more severe reduction in cholesterol efflux (19). Thus, genetic heterogeneity may exist, which may explain why patients with TD develop atherosclerosis (50, 51) and others do not (17). It is also possible that neither the HDL deficiency nor the reduced cholesterol efflux, nor the cholesterol ester deposition, are per se atherogenic, and that additional factors are necessary for atherogenesis. In addition, disturbances related to defective signal transduction in TD may be antiatherogenic. For example, our results indicate that the second messenger response induced by endothelin, which participates in the atherogenic process (52), might be impaired in TD cells. The markedly decreased LDL cholesterol levels in TD may also protect against atherogenesis.

During the last years the function and regulation of G-proteins and phospholipases has been intensively studied. However, little is known about the mechanisms, by which different phospholipases are acting together. We have identified a human disease, in which the G-protein–dependent coupling of two phospholipases is distorted. Our results should facilitate elucidating the molecular defect in TD and the identification of the protein(s) involved in the coupling between PC-PLC and PC-PLD and thus will help to understand the complex interplay between different phospholipases. In addition, our results demonstrate that the PC breakdown is essential for efficient HDL-mediated mobilization of cellular cholesterol. The coordinate formation of PA and DG via PC-PLD and PC-PLC seems to be necessary for this process.

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