Glycosphingolipid Accumulation Inhibits Cholesterol Efflux via the ABCA1/Apolipoprotein A-I Pathway

1-PHENYL-2-DECANOYLAMINO-3-MORPHOLINO-1-PROpanol IS A NOVEL CHOLESTEROL EFFLUX ACCELERATOR*

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Cellular glycosphingolipid (GSL) storage is known to promote cholesterol accumulation. Although physical interactions between GSLs and cholesterol are thought to cause intracellular cholesterol “trapping,” it is not known whether cholesterol homeostatic mechanisms are also impaired under these conditions. ApoA-I-mediated cholesterol efflux via ABCA1 (ATP-binding cassette transporter A1) is a key regulator of cellular cholesterol balance. Here, we show that apoA-I-mediated cholesterol efflux was inhibited (by up to 53% over 8 h) when fibroblasts were treated with lactosylceramide or the glucocerebrosidase inhibitor conduritol B epoxide. Furthermore, apoA-I-mediated cholesterol efflux from fibroblasts derived from patients with genetic GSL storage diseases (Fabry disease, Sandhoff disease, and GM1 gangliosidosis) was impaired compared with control cells. Conversely, apoA-I-mediated cholesterol efflux from fibroblasts and cholesterol-loaded macrophage foam cells was dose-dependently stimulated (by up to 6-fold over 8 h) by the GSL synthesis inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). Unexpectedly, a structurally unrelated GSL synthesis inhibitor, N-butyldeoxynojirimycin, was unable to stimulate apoA-I-mediated cholesterol efflux despite achieving similar GSL depletion. PDMP was found to up-regulate ABCA1 mRNA and protein expression, thereby identifying a contributing mechanism for the observed acceleration of cholesterol efflux to apoA-I. This study reveals a novel defect in cellular cholesterol homeostasis induced by GSL storage and identifies PDMP as a new agent for enhancing cholesterol efflux via the ABCA1/apoA-I pathway.

Previous work has established that cellular cholesterol trafficking and storage are linked to glycosphingolipid (GSL) metabolism (1–4). A clear example of this relationship is the demonstration that the cellular GSL storage detected in fibroblasts derived from patients with a variety of sphingolipid storage diseases (SLSDs) is accompanied by accumulation of endosomal/lysosomal cholesterol (1). Subsequent studies in human macrophage foam cells and fibroblasts indicated that GSL accumulation induced either by exogenously added lactosylceramide (LacCer) or by the glucocerebrosidase inhibitor conduritol B epoxide (CBE) results in concomitant accumulation of cholesterol (2, 4).

The factors that control the accumulation of cholesterol with GSLs remain to be identified. Purī et al. (1, 4) provided evidence supporting a “molecular trap” hypothesis, whereby increased GSL levels lead to increased cholesterol sequestration in late endosomes/lysosomes. Although the endosomal/lysosomal location of GSL and cholesterol in SLSDs is well known, it is becoming increasingly clear that cellular membrane composition is also altered when lysosomal storage of GSL/cholesterol occurs. Recent detailed studies by te Vruchte et al. (5) revealed that the accumulation of both GSL and cholesterol in ordered lipid microdomains commonly referred to as lipid rafts or, because of their relative resistance to detergent solubilization, detergent-resistant microdomains (DRMs) may contribute directly to lysosomal accumulation by altering endosomal transport pathways. These observations are consistent with previous proposals that alterations to membrane DRM structure could contribute to sphingolipidosis (6–8).

Although the co-localization of cholesterol with GSLs (4) and the accumulation of cholesterol and GSLs within DRMs (5) under GSL storage conditions support the GSL/cholesterol molecular trap concept (4), it is not known to what extent other pathways that regulate cellular cholesterol homeostasis are affected by GSL storage. Previous work has suggested that cholesterol that accumulates as a result of GSL storage may not be available to the sensing pathways that normally control intracellular cholesterol balance. Specifically, we have shown that macrophages induced to store cholesterol by treatment with LacCer or CBE down-regulate their apoE secretion (2). This is in contrast to the predicted up-regulation of apoE secretion in response to cholesterol loading (9). Similarly, Purī...
et al. (4) showed that low density lipoprotein (LDL) receptor expression is up-regulated when fibroblasts accumulate cholesterol as a consequence of LacCer treatment, again in contrast to the response predicted by Brown and Goldstein (10). The pathways that contribute to GSL-induced cellular cholesterol accumulation clearly require further study.

A key pathway regulating cellular cholesterol homeostasis relies on the apoA-I-mediated efflux of cholesterol from cells via ABCA1 (APTP-binding cassette transporter A1) (11). Cells from patients with genetic defects in ABCA1 leading to Tangier disease exhibit impaired cholesterol efflux to apoA-I and increased cellular cholesterol accumulation (12). Although it is clear that cellular GSL storage promotes cholesterol accumulation, it is not known whether alterations in the apoA-I-mediated cellular cholesterol efflux pathway are affected by GSL status. In this study, we therefore examined the impact of cellular GSL accumulation or depletion on apoA-I-mediated cholesterol efflux.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Cholesterol and [methyl-3H]choline chloride (specific activities of 44 and 79 Ci/mmol, respectively) were from Amersham Biosciences. 1-Palmitoyl-2-oleoylsphingosyldihexadecanol, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), N-butyldeoxynojirimycin (NB-DNJ), and CBE were from Sigma. LacCer was from Calbiochem. Human apoA-I was isolated from human high density lipoprotein (HDL) by ultracentrifugation and anion exchange chromatography (13).

**Cell Culture**—Human foreskin fibroblasts from a “control” subject and patients with GM1 gangliosidosis, Sandhoff disease, and Fabry disease (cell line identification numbers AG01518, GM01919, GM00203A, and GM004390, respectively) were obtained at passages 4–8 from the Coriell Institute (Camden, NJ) and grown under standard culture conditions at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 50 IU/ml streptomycin, 50 mg/ml gentamicin, 50 IU/ml amphotericin B, and 2 mM L-glutamine. The media were changed every 2–3 days. Control fibroblasts were cultured in tissue culture flasks and monolayers were maintained in flasks or on 35-mm plastic Petri dishes (Costar) for use in experiments. Fibroblasts were routinely used from passages 14 to 20. Human monocytes were isolated from donor buffy coats using ficoll-paque as described previously (14). Monocytes were allowed to differentiate into macrophages for 1 week in RPMI 1640 medium containing 10% (v/v) human serum supplemented with L-glutamine, penicillin, and streptomycin as above before further treatments. Cell viability was assessed by trypan blue exclusion.

**Lipoprotein Preparation**—LDL was isolated from normolipidemic plasma of 12-h fasted healthy subjects by ultracentrifugation (15). After purification, LDL was acetylated using an established method (16), and substituted for high density lipoprotein (HDL) by ultracentrifugation and anion exchange chromatography, and the glycan moiety was released by glycocerebrosidase addition (23). The GSL glycans were then fluorescently labeled with 2-imidazoyl-6-(biotinamido)ethyl-1,3-dithiopropionate (Pierce) for 30 min at 4 °C on a platform mixer. The biotinylation treatment was stopped with two washes of ice-cold quench buffer (50 mM Tris, 0.1 mM EDTA, and 150 mM NaCl), followed by a wash with ice-cold PBS. Cells were colabeled with fluorescent ceramide glycanase (12). Lysates were prepared as described previously (13). Protein concentration was determined using a bicinchoninic acid assay with bovine serum albumin as a standard. Lysates were pelleted by centrifugation and washed five times with lysis buffer. The resulting protein sample was analyzed by Western blotting for ABCA1 (as described above) or for scavenger receptor class B, type I (SR-BI) using a rabbit anti-mouse polyclonal antibody (1:1000 dilution; Novus Biologicals) that cross-reacts with human SR-BI.

**Cellular GSL Expression**—The GSL profiles of fibroblasts were analyzed as described previously (2, 23). Briefly, cells grown to confluency in 75-cm² flasks were rinsed three times with PBS and extracted in 2.1 (v/v) chloroform/methanol. The GSL fractions were isolated by liquid chromatography, and the glycan moiety was released by glycocerebrosidase addition (23). The GSL glycan was then fluorescently labeled with 2-iminobenzamide and analyzed by normal phase HPLC as described previously (24). Glycan peaks were identified by calculation of glucose units, derived from a partially hydrolyzed dextran standard, and comparison with previously published glucose units (2, 23). Cell-surface GSL expression levels were assessed by measuring the binding of fluorescently labeled cholera toxin B (CTxB) to cell-surface GM1 (10 min at 4 °C). Alexa Fluor 485-conjugated CTxB (Molecular
LacCer concentration to 70% versus 5.3% impact of such treatments on the ability of fibroblasts to efflux albumin (2–4). Here, we investigated the two-tailed standard (Sigma). unpublished data.

mediated cholesterol efflux was inhibited by 40% (8.9%). At the 8-h time point, apoA-I—present in the presence of albumin (Fig. 1A). ApoA-I, although the magnitude of this inhibition was not as marked as that seen for cholesterol (Fig. 2A). Although, the GM1 gangliosidosis cells were inefficient in cholesterol efflux under control conditions). Does Cellular GSL Accumulation Correlate with ABCA1 Expression?—Transcriptional regulation of ABCA1 expression is a key modulator of cholesterol efflux to apoA-I (29). To understand the factors that contribute to the impaired efflux of cholesterol from LacCer-treated cells, we used quantitative real-time PCR and Western blotting to analyze for expression of ABCA1. Treatment of fibroblasts with 40 μM LacCer did not significantly reduce ABCA1 mRNA levels (n = 4) overall.2 Similarly, total ABCA1 protein levels were only moderately reduced with LacCer treatment (Fig. 3A). The level of cell-surface ABCA1 expression was reduced, however, by as much as 50% after LacCer treatment (Fig. 3B). These data show that, although LacCer does not regulate ABCA1 expression directly, intracellular pathways, presumably related to ABCA1 trafficking or degradation, are significantly perturbed.

When the SLSD and control fibroblasts were compared, we found no correlation between the levels of ABCA1 mRNA expression and the rate of cholesterol efflux (Fig. 4A; cf. Fig. 1C). Although, the GM1 gangliosidosis cells were inefficient in cholesterol efflux activity and contained only one-third the level of ABCA1 mRNA compared with control fibroblasts, the Fabry disease fibroblasts expressed increased ABCA1 mRNA levels while still exhibiting a suppressed capacity to efflux cholesterol.

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terol esterification was significantly decreased under all conditions resulting in GSL accumulation (Fig. 4B). The relative levels of cholesterol esterification did not, however, strictly correlate with cholesterol efflux efficiency in the SLSD cells (Fig. 4B; cf. Fig. 1C). Therefore, the potential decrease in acyl-CoA:cholesterol acyltransferase-accessible cholesterol, which contributes directly to the pool that is effluxed to apoA-I (31), appears to contribute to varying degrees to the impaired cholesterol efflux associated with the different SLSDs.

We next investigated whether the impaired cholesterol efflux induced by GSL accumulation in the SLS Ds is associated more closely with a specific glycolipid species or total GSL load. HPLC analysis of control fibroblast GSLs revealed the major species as LacCer, ceramide trihexoside, and gangliosides GM3 and GM2, with a minor contribution made by GM1 (Fig. 5). In the SLS D cells, the GSL profiles were altered in concordance with the known genetic defects (i.e. Fabry disease, α-galactosidase defect resulting in elevated ceramide trihexoside levels; Sandhoff disease, β-hexosaminidase A/B defect resulting in elevated GM2/globoside levels; and GM1 gangliosidosis, β-galactosidase defect resulting in elevated GM1 levels). In the SLSD cells, additional GSLs were also markedly increased (e.g. LacCer and ceramide trihexoside in GM1 gangliosidosis and GM2 in Fabry disease) compared with control cells (Fig. 5A). Although cellular LacCer levels were increased to a variable extent in all of the SLSD cells examined, only the total cellular concentration of GSLs correlated (inversely) with apoA-I-mediated cholesterol efflux (Fig. 5B).

**Modulation of ApoA-I-dependent Cholesterol Efflux by PDMP**—Having established that increased cellular GSL levels suppressed cholesterol efflux, we wanted to know whether the reverse was also true, i.e. could a reduction in cellular GSL levels result in accelerated cholesterol efflux to apoA-I? To test
treated control fibroblasts with the GSL synthesis inhibitor PDMP. This compound inhibits glucosylceramide transferase activity, the initial step in GSL synthesis, thereby decreasing cellular levels of a range of GSLs, including glucosylceramide, LacCer, and GM1 (32–35). Confirming previous studies, treatment of fibroblasts with PDMP for 72 h inhibited cellular GSL levels as determined by binding of fluorescently labeled CTxB to cell-surface GM1 (Fig. 6). PDMP was initially added to subconfluent (60%) fibroblasts so that, after 72 h of incubation, the cells were confluent. At PDMP concentrations ≤ 40 μM, cell growth was inhibited as assessed by total protein measurements. At concentrations of 2.5–10 μM, there was no significant effect of the drug on cell protein, whereas GSL levels were reduced by 11 and 23%, respectively (Fig. 6). We therefore used PDMP at low concentrations to minimize potential growth inhibitory effects in subsequent analyses. PDMP significantly stimulated cholesterol efflux to apoA-I (Fig. 7). At 10 μM, PDMP increased cholesterol efflux to apoA-I by up to 6-fold (2.8 ± 0.8-fold increase above apoA-I alone, mean ± S.E., n = 7) as assessed at the 8-h time point while having no effect on basal efflux (Fig. 7). At 2.5 μM, PDMP increased efflux to apoA-I by 1.6 ± 0.1-fold (mean ± S.E., n = 3) at t = 8 h. The lack of effect on basal efflux implied that PDMP selectively promotes efflux via ABCA1. To confirm this, we assessed the effect of PDMP on cholesterol efflux to PLVs and MBCD, acceptors that do not require ABCA1 (27, 28). At 10 μM, PDMP did not affect cholesterol efflux to either PLVs or MBCD (Fig. 8). These data indicate that PDMP promotes cholesterol efflux through an ABCA1-dependent pathway.

**Effect of PDMP on ABCA1 Expression and Cholesterol Solubility**—At least two mechanisms could account for the effect of PDMP on apoA-I-dependent cholesterol efflux. First, the drug could up-regulate ABCA1 expression or activity either directly or via altered concentrations of an endogenous lipid mediator. Second, if molecular trapping of cholesterol within GSL-en-
riched lipid microdomains (also known as DRMs or rafts) occurs, one would predict that depletion of cellular GSL would lead to increased mobility of membrane cholesterol, which may therefore be more efficiently transported through ABCA1 (which is not raft-associated in fibroblasts (36, 37)). Analogous to the second mechanism, depletion of plasma membrane SM is thought to “release” cholesterol from ordered lipid microdomains, resulting in accelerated efflux to apoA-I (38), whereas accumulation of cellular SM (as occurs in sphingomyelinase-deficient cells) “traps” cholesterol, thereby inhibiting efflux to apoA-I (30). We therefore conducted experiments to assess these possibilities. PDMP dose-dependently increased fibroblast ABCA1 mRNA levels (Fig. 9A). At 10 μM, PDMP increased ABCA1 mRNA levels by up to 2.7-fold (2.1 ± 0.3-fold, mean ± S.E., n = 3; p < 0.05). There was an overall trend toward increased ABCA1 mRNA expression at 2.5 μM, but this was not statistically significant in all experiments (1.3 ± 0.3-fold, mean ± S.E., n = 3). We also detected moderate increases in ABCA1 protein levels by Western blotting, but only at the higher PDMP dose (Fig. 9B). The levels of cell-surface ABCA1 expression were increased to a similar degree by PDMP, indicating that PDMP acts primarily by regulating ABCA1 expression and not by modulating trafficking to the cell surface (Fig. 9C). Overall, these data suggest that at least part of the effect of PDMP on cholesterol efflux is mediated through effects on ABCA1 expression.

To assess the potential for PDMP to alter cholesterol distribution in DRMs, we measured cholesterol partitioning in Triton-soluble and Triton-insoluble fractions using an established method (3, 25). Using cold 1.0% (w/v) Triton X-100 extraction, we found that ~25% of the [3H]cholesterol was insoluble (Fig. 10). There was dose-dependent trend for PDMP to reduce the amount of cholesterol present in the Triton-insoluble fraction; and at a concentration of 10 μM, ~21% of the cholesterol was insoluble (Fig. 10). At 40 μM PDMP, only 13% of the cholesterol remained insoluble, and this difference was statistically significant (Fig. 10B). Although the trend for 10 μM PDMP to reduce cholesterol insolubility appears modest, a potential contribution to accelerated efflux cannot be ruled out. These data show that, under the conditions in which PDMP accelerated apoA-I-mediated cholesterol efflux, a trend toward increased cholesterol solubility was also observed. This indicates that PDMP may promote the release of cholesterol from within DRMs, consistent with previous work showing that PDMP treatment increases the mobile fraction of synthetic lipid probes in the plasma membrane (39).

Lack of Effect of NB-DNJ on ApoA-I-Mediated Cholesterol Efflux—The above data suggest that PDMP may exert its action both through alterations in ABCA1 expression (or activity) and via reducing the physical trapping of cholesterol by GSLs within DRMs. Because compounds that promote cellular cholesterol efflux may be clinically useful as accelerators of reverse cholesterol transport (40), we conducted additional experiments in an attempt to further separate the effect of PDMP on ABCA1 expression from its moderate impact on the implied physical interaction of GSL with cholesterol in DRMs. To do this, we employed a structurally distinct GSL synthesis inhibitor, NB-DNJ, which also inhibits glucosylceramide transferase activity (41). We conducted dose-response experiments in the range of 10–500 μM NB-DNJ to achieve a level of GSL synthesis inhibition comparable to that observed with 10 μM PDMP without adversely affecting cell growth or morphology. Based on the CTxB binding assay, 50 μM NB-DNJ reduced GSL levels by 43% (Fig. 11A). This level of GSL depletion was more effective than that achieved using PDMP at subcytostatic concentrations (Fig. 11A; cf. Fig. 6). Similar to our experiments with PDMP, 50 μM NB-DNJ reduced the amount of Triton-insoluble...
cholesterol from 27 to 16% (Fig. 11A). Although NB-DNJ reduced cellular GSL levels and also increased cholesterol solubility to an extent even greater than observed with PDMP treatment, it did not significantly promote apoA-I-dependent cholesterol or phospholipid efflux (Fig. 11). This indicates that, under normal physiological conditions (in the absence of GSL storage), a reduction in cellular GSL levels per se does not appear to promote cholesterol efflux to apoA-I.

**PDMP Promotes ApoA-I-dependent Cholesterol Efflux from Human Monocyte-derived Macrophages**—Our studies using NB-DNJ suggest that PDMP may promote cholesterol efflux to apoA-I via a pathway that is not reliant on its ability to inhibit GSL synthesis. Although GSL synthesis inhibition per se may not be sufficient to induce cholesterol efflux, the fact that PDMP selectively promoted apoA-I-dependent cholesterol efflux could offer a new approach to promote reverse cholesterol transport in vivo. This would be of therapeutic interest for the treatment of atherosclerosis, where large amounts of cholesterol accumulate within macrophage foam cells in the arterial intima (42). We therefore assessed the impact of PDMP on apoA-I-mediated cholesterol efflux from human MDMs and acLDL-loaded MDM foam cells. At 10 μM, PDMP increased cholesterol efflux from MDMs and MDM foam cells to apoA-I by ~2-fold as assessed at the 8-h time point (Table 1). Interestingly, although PDMP did not significantly promote basal cholesterol efflux from fibroblasts, it did increase basal efflux from both MDMs and acLDL-loaded MDM foam cells. The effect of PDMP on basal cholesterol efflux is potentially related to the high levels of apoe secreted by MDMs, which could offer an additional cholesterol efflux vehicle (43). We have shown previously that CBE inhibits macrophage foam cell apoE secretion while promoting cholesterol accumulation (2). Here, we also assessed the impact of CBE treatment on apoA-I-mediated cholesterol efflux. Similar to the effect of CBE on fibroblasts,

**Fig. 10. Effect of PDMP on Triton-soluble and Triton-insoluble cholesterol distribution.** A, fibroblasts were grown in DMEM and 10% FCS with 0.2 mCi/ml [3H]cholesterol (3H-Chol.) or the same medium supplemented with 2.5, 10, or 40 μM PDMP for 72 h. Cells were then washed and equilibrated (in the presence of PDMP where appropriate) for 16 h. The cells were extracted into cold Triton X-100, and the detergent-insoluble (Insol.; ○) and detergent-soluble (Sol.; ○) cholesterol levels were measured as described under Experimental Procedures. B, the data are expressed as percent insoluble cholesterol. Values are the means ± S.E. from triplicate cultures. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (significantly different from control conditions).

**Fig. 11. Effect of NB-DNJ on fibroblast GSL levels, cholesterol solubility, and apoA-I-mediated cholesterol efflux.** A, fibroblasts were grown in DMEM and 10% FCS with [3H]cholesterol (3H-Chol.) or [3H]choline in the presence of 50 μM NB-DNJ (cross-hatched) as indicated for 72 h. Cells were then washed and equilibrated (in the presence of NB-DNJ where appropriate) for 16 h. The cells were assayed for cell-surface GM1 expression by CTxB binding, Triton X-100-insoluble cholesterol (TX-100), or 8-h [3H]cholesterol efflux in DMEM containing 0.1% (w/v) BSA (control (Con)) or in the same medium with the addition of 25 μg/ml apoA-I as described under Experimental Procedures. B, the effect of NB-DNJ on phospholipid efflux to apoA-I was analyzed. Values are the means ± S.E. from triplicate cultures, representative of two experiments, p < 0.05; ***, p < 0.01 (significantly different from parallel conditions without NB-DNJ treatment).

treatment of MDMs with 50 μM CBE inhibited subsequent cholesterol efflux to apoA-I (7.1 ± 0.82% versus 3.7 ± 0.38%, mean ± S.E., n = 3; p < 0.05) as assessed at the 8-h time point. CBE treatment also tended to increase total cellular cholesterol counts at t = 0 h by ~20%, but this was not statistically significant (244 ± 10) × 10^3 cpm/well versus (298 ± 21) × 10^3 cpm/well, mean ± S.E., n = 3; p = 0.09).

**DISCUSSION**

Our studies show for the first time that cellular LacCer accumulation or agents/genetic diseases that cause GSL storage inhibit apoA-I-mediated cholesterol efflux. These findings indicate that the cholesterol accumulation that accompanies GSL storage in SLSd is compounded by a reduced capacity of these cells to divest themselves of excess cholesterol. Interestingly, certain SLSdS are accompanied by low plasma HDL cholesterol levels. For example, Gaucher disease (patients have a β-glucocerebrosidase deficiency, and the condition is therefore mimicked by CBE in vitro) is accompanied by low plasma HDL cholesterol levels, which are increased toward normal levels with enzyme replacement therapy (44). Our experiments using CBE indicate that this may due to alterations in the ABCA1/apoA-I cholesterol efflux pathway. Patients with Niemann-Pick disease (types A and B) are deficient in acid sphingomyelinase activity and also have low plasma HDL cholesterol levels (45). Furthermore, cells derived from sphingomyelinase knockout mice are defective in their ability to efflux cholesterol to apoA-I, illustrating that sphingolipid storage can also inhibit cholesterol efflux (30). Patients with Niemann-Pick disease type C (deficiency in NPC1 or HE1/NPC2, proteins that regulate intracellular cholesterol trafficking) also have low plasma HDL cholesterol lev-
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Human monocytes were isolated from donor buffy coats and differentiated to MDMs for 1 week in 10% (v/v) human serum. MDMs from Donor 1 were then labeled with [3H]cholesterol in growth medium, whereas MDMs from Donor 2 were loaded with [3H]cholesterol-labeled acLDL (50 μg/ml protein). Under both labeling conditions, cells were also incubated in the presence or absence of 10 μM PDMP. Cholesterol efflux was assessed after a 6-h incubation with 25 μg/ml apo-A-I, and counts in cell supernatants are expressed as a percentage of the total. The presence of PDMP significantly reduced [3H]cholesterol counts in MDMs at r = 0 h by 30% and 35% in the experiments for Donors 1 and 2, respectively.

Consistent with this, in fibroblasts treated with 10 μM PDMP, we also detected a 2-fold increase in apoE mRNA levels and an 8-fold increase in ABCG1 mRNA levels by real-time PCR.2 Both of these genes are transcriptionally regulated by LXRα and protein levels. The mechanisms responsible for this increase in ABCA1 expression are unknown. One possibility is that PDMP treatment induces LXRα-dependent transcription either directly or via increased synthesis of an oxysterol ligand. Consistent with this, in fibroblasts treated with 10 μM PDMP, we also detected a 2-fold increase in apoE mRNA levels and an 8-fold increase in ABCG1 mRNA levels by real-time PCR.2 Both of these genes are transcriptionally regulated by LXRα (9, 21, 52). Although these changes are not likely to contribute to the increased cholesterol efflux to apoA-I observed (as ABCG1 does not promote efflux to apoA-I (53), and we did not detect secretion of apoE by fibroblasts), it does indicate that other LXRα-regulated genes are induced.

Regardless of the mechanisms operating in the different SLSDs, our data indicate that the impaired capacity of SLSD cells to release cholesterol to apoA-I may contribute to the reduced HDL levels observed in specific GSL storage conditions in vivo. This could ultimately promote additional GSL storage through cellular GSL efflux (2). Our data, in addition to the studies cited above, indicate that the association of impaired ABCA1-mediated cholesterol efflux with SLSDs likely contributes to the storage phenotype.

Previous studies indicated that cellular cholesterol efflux is inhibited by SM accumulation and accelerated when SM is depleted by the addition of sphingomyelinase to cells in vitro (30, 38, 48). Although these observations appear to be analogous to our findings with cellular GSL accumulation and depletion (via PDMP treatment), important differences exist. Depletion of plasma membrane SM appears to release cholesterol from ordered lipid microdomains, resulting in accelerated efflux to apoA-I (38), whereas accumulation of cellular SM (as occurs in sphingomyelinase-deficient cells) traps cholesterol, thereby inhibiting efflux to apoA-I (30). Although there may be mechanistic similarities when either SM or GSL levels are increased, leading to cholesterol trapping (as suggested previously (1, 7)), depletion of cellular GSL was not sufficient to promote cholesterol efflux, as demonstrated by our experiments with NB-DNJ (Fig. 11).

The use of cold detergent extraction provides a facile approach to detect gross changes in membrane lipid order (25, 47). Although we detected an increase in Triton-soluble cholesterol when GSL depletion was induced with NB-DNJ, this alone was insufficient to promote apoA-I-mediated cholesterol efflux. It is not clear why plasma membrane SM depletion stimulates apoA-I-mediated cholesterol efflux (38), whereas GSL depletion induced by NB-DNJ does not, as both sphingolipids form complexes with cholesterol (Ref. 49 and references cited therein). The magnitude of change in cholesterol solubility we detected may be insufficient to have an impact on apoA-I-mediated cholesterol efflux, i.e. in the studies of Ito et al. (38), sphingomyelinase treatment decreased 0.1% Triton X-100-insoluble cholesterol levels by ~50%. In addition, where GSL levels were chronically reduced in our studies, compensatory pathways (such as increased saturation of SM and glycerophospholipid acyl chains) may be induced to maintain membrane lipid order (50) and thereby minimize alterations in cholesterol solubility.

* p < 0.01 (significantly different from appropriate control conditions without PDMP), ** p < 0.05 (significantly different from appropriate control conditions without PDMP).

| Donor | Control | PDMP | Apo-A-I | Apo-A-I + PDMP |
|-------|---------|------|--------|---------------|
| Donor 1 | 3.5 (0.20) | 8.7 (0.19)** | 9.4 (0.15) | 18.6 (0.36)** |
| Donor 2 | 4.9 (0.94) | 9.1 (0.63)** | 8.8 (0.41) | 19.4 (0.86)** |

TABLE I Acceleration of apoA-I-mediated cholesterol efflux from human MDMs and acLDL-loaded MDM foam cells by 10 μM PDMP.
arterial lesions in humans and in animal models of the disease (58–60). Previous studies have shown that LacCer is proatherogenic, e.g. by acting as a smooth muscle cell mitogen (61, 62); however, its impact on apoA-I-mediated cholesterol efflux was not recognized. Numerous studies have focused on the mechanisms resulting in cholesterol accumulation in atherosclerotic lesions, principally within MDM foam cells (42). Our discovery that PDMP promotes cellular cholesterol efflux to apoA-I and reduces GSL levels (processes that appear to be independent of each other) suggests that PDMP or structurally related compounds may represent a novel approach to treat atherosclerosis.

In conclusion, our studies show for the first time that cellular GSL accumulation inhibits apoA-I-mediated cholesterol efflux. Our studies also reveal PDMP as a novel cholesterol efflux accelerator. This may provide a useful approach to promote apoA-I-mediated efflux and reverse cholesterol transport in vivo.

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