Lysosomal glycosidases and glycosphingolipids: New avenues for research

Rosa Alcalde Marques, A.

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CHAPTER 13

Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases

Chapter 7

Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases

André R. A. Marques1,#; Mina Mirzaian2,#; Hisako Akiyama3,#; Patrick Wisse4; Maria J. Ferraz1; Paulo Gaspar1; Karen Ghauharali-van der Vlugt 1; Rianne Meijer 2; Pilar Giraldo5; Pilar Alfonso 5; Pilar Irún 5; Maria Dahl6; Stefan Karlsson6; Elena V. Pavlova7; Timothy M. Cox7; Saskia Scheij1; Marri Verhoek 2; Roelof Ottenhoff1; Cindy P. A. A. van Roomen 1; Navraj S. Pannu 2; Marco van Eijk 2; Nick Dekker1; Rolf G. Boot2; Herman S. Overkleeft4; Edward Blommaart1; Yoshio Hirabayashi3; Johannes M. Aerts1,2

1Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands; 2Department of Medical Biochemistry, Leiden Institute of Chemistry, Leiden, The Netherlands; 3Brain Science Institute, RIKEN, Wako, Japan; 4Department of Bio-organic Synthesis, Leiden Institute of Chemistry, Leiden, The Netherlands; 5Centro de Investigación Biomédica en Red de Enfermedades Raras, Unidad de Investigación Traslacional, Zaragoza, Spain; 6Department of Molecular Medicine and Gene Therapy, Lund University, Lund, Sweden; 7Addenbrooke’s Hospital, Department of Medicine, University of Cambridge, Cambridge, UK

#These authors contributed equally to this work, and should be considered as first authors.

Abstract

The membrane lipid glucosylceramide (GlcCer) is continuously formed and degraded. Cells express two GlcCer-degrading β-glucosidases, GBA and GBA2, located in and outside the lysosome, respectively. Here we demonstrate that through transglucosylation both GBA and GBA2 are able to catalyze in vitro the transfer of glucosyl-moieties from GlcCer to cholesterol, and vice versa. Furthermore, the natural occurrence of 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) in mouse tissues and human plasma is demonstrated using LC-MS/MS and 13C6-labelled GlcChol as internal standard. In cells the inhibition of GBA increases GlcChol, whereas inhibition of GBA2 decreases glucosylated sterol. Similarly, in GBA2-deficient mice GlcChol is reduced. Depletion of GlcCer by inhibition of GlcCer synthase decreases GlcChol in cells and likewise in plasma of inhibitor-treated Gaucher disease patients. In tissues of mice with Niemann-Pick type C, a condition characterized by intralysosomal accumulation of cholesterol, marked elevations in GlcChol occur as well. When lysosomal accumulation of cholesterol is induced in cultured cells, GlcChol is formed via lysosomal GBA. This illustrates that reversible transglucosylation reactions are highly dependent on local availability of suitable acceptors. In conclusion, mammalian tissues contain GlcChol formed by transglucosylation through β-glucosidases using GlcCer as donor. Our findings reveal a novel metabolic function for GlcCer.

Keywords: cholesterol, glucosyl-β-cholesterol, glucosylceramide, glucocerebrosidase, Gaucher disease, Niemann Pick type C disease.
Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases

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Abstract The membrane lipid glucosylceramide (GlcCer) is continuously formed and degraded. Cells express two GlcCer-degrading β-glucosidases, GBA and GBA2, located in and outside the lysosome, respectively. Here we demonstrate that through transglucosylation both GBA and GBA2 are able to catalyze in vitro the transfer of glucosyl-moieties from GlcCer to cholesterol, and vice versa. Furthermore, the natural occurrence of 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) in mouse tissues and human plasma is demonstrated using LC-MS/MS and $^{13}$C$_6$-labelled GlcChol as internal standard. In cells the inhibition of GBA increases GlcChol, whereas inhibition of GBA2 decreases glucosylated sterol. Similarly, in GBA2-deficient mice GlcChol is reduced. Depletion of GlcCer by inhibition of GlcCer synthase decreases GlcChol in cells and likewise in plasma of inhibitor-treated Gaucher disease patients. In tissues of mice with Niemann-Pick type C, a condition characterized by intralysosomal accumulation of cholesterol, marked elevations in GlcChol occur as well. When lysosomal accumulation of cholesterol is induced in cultured cells, GlcChol is formed via lysosomal GBA. This illustrates that reversible transglucosylation reactions are highly dependent on local availability of suitable acceptors. In conclusion, mammalian tissues contain GlcChol formed by transglucosylation through β-glucosidases using GlcCer as donor. Our findings reveal a novel metabolic function for GlcCer.

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Introduction

Membranes of higher eukaryotic cells contain glycerolipids, sterols and sphingolipids. For each of these lipid classes, monoglucosylated structures have been reported. Glucosylceramide (GlcCer), the intermediate in biosynthesis and degradation of more complex glycosphingolipids (GSLs), is ubiquitous in mammalian cells, particularly located in the cell membrane (1). Its presence in plants and some fungi is also documented. Glucosyldiacylglycerol (GlcDG) has been identified in various plants, but its presence in mammalian cells is comparatively poorly documented (2, 3). Likewise, sterol-glucosides are known to occur in plants and fungal species (4), but their existence in mammalian cells has not been extensively studied. Indications of the existence of glucosyl-β-D-cholesterol or 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) in mammalian cells were first provided by Murofushi and co-workers. They described its occurrence in cultured human fibroblasts and gastric mucosa (5, 6). Heat shock was found to increase biosynthesis of GlcChol and subsequently induce HSP70 (7). GlcCer is formed by the enzyme glucosylceramide synthase (GCS, EC2.4.1.80). This transferase, firstly cloned by Hirabayashi and colleagues (8), is located at the endoplasmic reticulum (ER) in hepatocytes (22), at the ER and Golgi apparatus in neuronal cells (23). GBA2 degrades GlcCer without need for an activator protein, and further differs from GBA in noted at the endoplasmic reticulum (24). GBA2 has been found to be located outside lysosomes, being referred to as broad-specific cytosolic glucosylceramidase (21).

The enzyme GBA is well studied since its deficiency underlies Gaucher disease (GD), a relatively common lysosomal storage disease (LSD) (13). Assisted by the small activator protein saposin C, GBA degrades GlcCer to ceramide and glucose in lysosomes, the penultimate step in GSL catabolism (13). Deficient GBA activity in GD patients consequently results in accumulation of GlcCer in lysosomes, most prominently in macrophages. These “Gaucher cells” secrete specific proteins as well as glucosylphosphingosine (GlcSph), the deacylated form of GlcCer (14–16). The non-neuronopathic (type 1) variant of GD is presently treated by enzyme replacement therapy (ERT), implying chronic two-weekly intravenous infusion of macrophage-targeted recombinant enzyme (17). An alternative treatment of type 1 GD, named substrate reduction, is based on oral administration of an inhibitor of GCS (18–20).
Mammalian cells and tissues contain other β-glucosidases besides GBA that degrade GlcCer. All cells express the membrane-associated non-lysosomal glucosylceramidase, named GBA2 (21–23). This enzyme is not deficient in GD patients. In fact, a compensatory overexpression of GBA2 in materials of GD has been reported (24). GBA2 has been found to be located outside lysosomes, being noted at the endoplasmic reticulum (ER) in hepatocytes (22), at the ER and Golgi apparatus in HEK293 cells overexpressing enzyme (25) and at the endosomes in fibroblasts and COS-7 cells (23). GBA2 degrades GlcCer without need for an activator protein, and further differs from GBA in noted artificial substrate and inhibitor specificity (21). Finally, some tissues express the enzyme GBA3, also referred to as broad-specific cytosolic β-glucosidase (26). This enzyme shows a relative poor in vitro hydrolytic activity towards GlcCer and is thought to be primarily involved in de-toxification of glucosylated xenobiotics (26). All three human retaining β-glucosidases employ the double displacement mechanism in catalysis. There are many documented examples of transglucosylation mediated by retaining glycosidases (27). Therefore, in addition to GBA, theoretically also GBA2 and GBA3 might generate GlcChol.

Modification of cholesterol by glucosylation changes the physico-chemical properties of the sterol, rendering it far more water soluble. Given the potential physiological relevance, the natural occurrence of GlcChol, and its metabolism, in cells and tissues is of interest. We therefore studied the existence of the glucosylated sterol in mammalian tissues. For this purpose 13C₆-isotope labelled GlcChol was synthesized to be used as internal standard in sensitive quantitative detection of GlcChol by LC-MS/MS. Here we demonstrate the natural occurrence of GlcChol in mammalian cells and tissues. Moreover we document the ability of both GBA and GBA2 to degrade as well as synthesize GlcChol. The importance of substrate and acceptor concentrations regarding the action of GBA and GBA2 in GlcChol metabolism is experimentally demonstrated. Our investigation demonstrates the surprising versatility of β-glucosidases, a finding discussed in relation to metabolism of sphingolipids and sterols in health and disease.

**Materials and Methods**

**Materials**  25-[(N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (25-NBD-Cholesterol), N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino[hexanoyl]-D-glucosyl-β-1′-sphingosine (C6-NBD-GlcCer), N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino[hexanoyl]-D-erythro-sphingosine (C6-NBD-Cer), D-glucosyl-β-1,1′ N-palmitoyl-D-erythro-sphingosine (C16:0-GlcCer) and D-glucosyl-β-1,1′ N-oleoyl-D-erythro-sphingosine (C18:1-GlcCer) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 4-methylumbelliferyl β-D-gluco pyranoside (4MU-Glc) was purchased from Glycosynth™ (Winwick Quay Warrington, Cheshire, England). Conduritol B epoxide (D, L-1,2-anhydro-my o-inositol; CBE) was from Enzo Life Sciences Inc. (Farmingdale, NY, USA), 1-O-cholesterol-β-D-glucopyranoside (β-cholesterol glucoside, β-GlcChol) and ammonium formate (LC-MS quality) were from Sigma-Aldrich (St Louis, MO, USA). N-(n-Butyl)deoxygalactonojirimycin (NB-DGJ) was purchased from Toronto Research Chemicals (Toronto, Canada). GBA2 inhibitor N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) and GBA3 inhibitor α-1-C-nonyl-DIX (anDIX) were chemically synthesized in the department of Bio-organic Synthesis at the Faculty of Science, Leiden Institute of Chemistry at the University of Leiden (Leiden, The Netherlands). Cerzyme®, a recombinant human GBA (rGBA) was obtained from Genzyme (Genzyme Nederland, Naarden, The Netherlands). Cholesterol trafficking inhibitor U18666A and methyl-β-cyclodextrin were from Sigma-Aldrich Chemie GmbH. LC-MS–grade methanol, 2-propanol, water, HPLC-grade chloroform were purchased from Biosolve; ammonium formate LC-MS grade from Sigma-Aldrich Chemie GmbH.

Synthesis of 13C₆ isotope labelled β-cholesterol glucoside (13C₆-β-GlcChol) The synthesis of 13C-labelled glucosyl donor 4 (see Scheme 1) commences with protecting the five hydroxyls in glucose 1 as the benzoyl
esters using pyridine and benzoil chloride to give 1,2,3,4,6-penta-O-benzoyl-$\beta$-D-$^{13}$C$_6$-glucopyranoside 2 quantitatively. In the next step the anomeric benzoate was selectively removed using hydrazine acetate providing 2,3,4,6-tetra-O-benzoyl-$\alpha$/$\beta$-D-$^{13}$C$_6$-glucopyranoside 3 in 82% yield. The anomeric hydroxyl in 3 was transformed into the corresponding trichloroacetimiclidate using trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene as base giving 2,3,4,6-tetra-O-benzoyl-$\alpha$-(2,2,2-trichloroethanamidate)-$\alpha$-D-$^{13}$C$_6$-glucopyranoside 4. In the penultimate step, cholesterol was reacted with 4 under the agency of a catalytic amount of trimethylsilylmethanesulphonate (TMSOTf) in dichloromethane at room temperature. After 1 h the reaction was quenched with triethylamine and the mixture purified by silica gel column chromatography giving cholesteryl 2,3,4,6-tetra-O-benzoyl-$\beta$-D-$^{13}$C$_6$-glucopyranoside 5 in 83%. Compound 5 was deprotected using sodium methoxide in methanol/dichloromethane giving after silica gel column chromatography the title compound, cholesteryl-$\beta$-D-$^{13}$C$_6$-glucopyranoside ($^{13}$C$_6$-GlcChol) 6 as a white solid in 94%.

Scheme 1. Synthesis of cholesteryl-$\beta$-D-$^{13}$C$_6$-glucopyranoside ($^{13}$C$_6$-GlcChol) 6 (28).

**Animal Studies**

Npc1$^{+/+}$ mice (Npc1$^{inh}$ and Npc1$^{pm}$), along with wild-type (wt) littermates (Npc1$^{+/+}$), were generated by crossing Npc1$^{+/+}$ males and females in-house. The heterozygous BALB/c Ncr-Npc1$^{inh}$ mouse (stock number 003092) and heterozygous C57BLKS/J-Npc1$^{pm}$/J mouse (stock number 002760) were obtained from the Jackson Laboratory (Bar Harbor, USA). Mouse pups were genotyped according to published protocols (29, 30). The Gba2$^{+/+}$ mice (C57Bl/6-129S6/SvEv mixed background) were generated as previously described (22). Breeding pairs of LIMP-2 were kindly provided by Prof. Paul Saftig (Kiel, Germany) (31). Homozygous WT animals (LIMP2$^{+/+}$) and homozygous animals (LIMP2$^{-/-}$) were generated by crossing heterozygous (LIMP2$^{+/+}$) mice. Genotyping was determined by PCR using genomic DNA (31). Mice (± 3 weeks old) received the rodent AM-II diet (Arie Blok Diervoeders, Woerden, The Netherlands). The mice were housed at the Institute Animal Core Facility in a temperature- and humidity-controlled room with a 12-h light/dark cycle and given access to food and water ad libitum. All animal protocols were approved by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam in the Netherlands (DBC101698, DBC100757-115, DBC100757-125 and DBC17AC).

The generation of the GD1 mouse model has been described previously (32, 33). Mice were maintained in individually ventilated cages with ad libitum food and water in the animal facility at Lund University Biomedical Center. Breeding and experimental procedures were approved by the Committee for Animal Ethics in Malmö/Lund, Sweden.

Animals were first anesthetized with a dose of Hypnorm (0.315 mg/mL phenyl citrate and 10 mg/mL fluanisone) and Dormicum (5 mg/mL midazolam) according to their weight. The given dose was 80 µL/10 g bodyweight. Animals were sacrificed by cervical dislocation. Organs were collected by surgery, rinsed with PBS, directly snap-frozen in liquid nitrogen and stored at -80 °C. Later, homogenates were made from the frozen material in 25 mM potassium phosphate buffer pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitors (4 µL of buffer per mg of tissue).
Cloning of cDNAs encoding GBA2, GBA3 and UGCG The design of cloning primers was based on NCBI reference sequences NM_172692.3 for murine GBA2, NM_020973.3 for human hGBA3 and NM_003358.2 for human UGCG (GCS). Using the primers listed below, the full-length coding sequences were cloned into pcdNA3.1/Myc-His (Invitrogen, Life Technologies, Carlsbad, CA, USA), using primers: RB143: GAATTCCGGCCACCATGGAACCTGCCTCGGG and RB144: GCGGCCGCTCTGAAATCCACCTGCCCAG for mGBA2; RB252: GAATTCCGGCCACCATGGTTCGCTCAGGATTTG and RB253: GCGGCCGCTACAGATGTTCAAGGCC for hGBA3; RB111: TCCTGCAGGAGCGTTCGTC and RB114: GGTACCATCATCTAGATTCTCCTGCG for hUCGC. These constructs were used to transfect COS-7 cells.

For the transfection of Chinese hamster ovary cells (CHO-K1 cells) full-length coding sequence for transcript variant 1 of human GBA3 (NM_020973.3) was cloned into p3xFLAG-CMV-14 (Sigma-Aldrich, St. Louis, MO, USA) as described previously (11).

Cell culture and transfection RAW264.7 cells were obtained from the “American Type Culture Collection” and were cultured in DMEM (Dulbecco’s Modified Eagle Medium; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands) with penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). COS-7 cells were cultured in Iscove’s modified Dulbecco’s medium with 5% FBS and penicillin/streptomycin under 5% CO2 at 37°C. Cells were seeded at 75% confluence in 6-well plates and transfected using FuGENE® 6 Transfection Reagent (Promega Benelux, Leiden, The Netherlands) according to the manufacturer’s instructions, at a FuGENE:DNA ratio of 3:1. After 24 h, inhibitors of GBA (CBE, 300 µM) or GBA2 (AMP-DNM, 20 nM) were added and 48 h later, the medium was removed, cells were washed twice with ice-cold PBS and harvested by scraping in 25 mM potassium-phosphate buffer pH 6.5. CHO-K1 cells (RCB2085, established by Puck, T. T.) were purchased from RIKEN BioResource Center (Ibaraki, Japan) and cultured in Ham’s F-12 medium (Nissui) supplemented with 10% FBS under 5% CO2 at 37°C. CDNA transfection for CHO-K1 cells was carried out using Lipofectamine® 2000 Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 24 h, medium containing transfection reagents was removed, and cells were incubated with lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 tablet/10 mL Complete Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland), pH 7.4] for 15-30 min at 4°C after washing with PBS. The cells were harvested and centrifuged at 12,000xg for 10 min at 4°C. The obtained supernatants were collected for in vitro enzyme assays.

In vitro assay of transglucosylase activity Lysates of COS-7 cells overexpressing GBA2, GBA3, GCS, and rGBA were used to determine transglucosylase activity of each enzyme. The assay was performed as described earlier (11) with a few modifications. First, 40 µL of homogenate of cells overexpressing GBA2, GBA3 or GCS was pre-incubated with 10 µL of 25 mM CBE in water for 20 min (samples containing diluted rGBA were pre-incubated in the absence of CBE). To each of the samples 200 µL of the appropriate buffer containing 100 µM of donor (either C18:1-GlcCer or GlcChol) and 40 µM of acceptor (either 25-NBD-Cholesterol or C6-NBD-Cer), was added. Transglucosylase activity of GBA2 overexpressing cells was measured in a 150 mM McIlvaine buffer pH 5.8 and the assay for rGBA was done in a 150 mM McIlvaine buffer pH 5.2 containing 0.1% BSA, 0.1% Triton X-100 and 0.2% sodium taurocholate. For GBA3 the assay contained 100 mM HEPES buffer, pH 7.0. The transglucosylase assay for GCS was performed in a 125 mM potassium-phosphate buffer pH 7.5 with 12.5 mM UDP-glucose, 6.25 mM MgCl2, 0.125% BSA, and 0.625% CHAPS. After 1 h of incubation at 37°C, the reaction was terminated by addition of chloroform/methanol (1:1, v/v) and lipids were extracted according to Bligh and Dyer (34). Thereafter lipids were separated by TLC on HPTLC silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol (85:15, v/v) as eluent followed by detection of NBD-labelled lipids using a Typhoon Variable Mode Imager (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) (35).

Identification of newly formed fluorescent lipid in transglucosylation assays with 25-NBD cholesterol as acceptor was performed following its isolation by scraping from plates by demonstration of complete digestion to NBD-cholesterol using excess rGBA at pH 5.2 (McIlvaine buffer) in the presence of 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100.
Lysates of CHO-K1 cells were used to access the transglucosylase activity and the β-glucosidase activity of GBA3. The assay for transglucosylase activity was performed according to the method we established previously (11) with slight modifications. The reaction mixture in a total volume of 20 μL contained 40 μM 25-NBD-cholesterol, 80 μM C16:0-GlcCer, 50 mM citrate-phosphate buffer, pH 6.2, 0.5% CHAPS, 2% ethanol, and desired amount of enzyme. After incubation at 37°C for 20 h, the reaction was terminated by adding chloroform/methanol (2:1, v/v), and the lipids were extracted and analyzed as reported before (11). The assay for β-glucosidase activity was performed according to the method we established previously (11) with slight modifications. The reaction mixture in a total volume of 20 μL contained 100 pmol C6-NBD-GlcCer, 50 mM citrate-phosphate buffer, pH 6.2, and a desired amount of enzyme. After incubation at 37°C for 30 min, the reaction was terminated by adding chloroform/methanol (2:1, v/v), and the lipids were extracted and analyzed as reported before (11).

Analysis of GlcChol by LC-MS/MS A Waters Acquity™ TQD instrument was used in all experiments. The instrument consisted of a UPLC system combined with a tandem quadruple mass spectrometer as mass analyzer. Data were analyzed with Masslynx 4.1 Software (Waters Corporation; Milford MA). GlcChol and $^{13}$C$_6$-GlcChol (internal standard) were separated using a BEH C18 reversed-phase column (2.1x 50 mm, particle size 1.7 μm; Waters), by applying an isocratic elution of mobile phases, 2-propanol:H$_2$O 90:10 (v/v) containing 10 mM ammonium formate (Eluent A) and methanol containing 10 mM ammonium formate (Eluent B). The ULPC program was applied during 5.0 minutes consisting of 10% A and 90% B. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0 to 0.25 min) and after (4 to 5 min) the elution of the analytes to prevent system contamination. The flow rate was 0.250 mL/min and the retention time of both GlcChol and the internal standard was 1.43 min (Figure 1C). The column temperature and the temperature of the autosampler were kept at 23°C and 10°C respectively during the run.

Solutions of GlcChol and $^{13}$C$_6$-GlcChol and a mixture of both compounds were prepared with concentrations of 1 pmol/μL in 5 mM ammonium formate in methanol. The compounds were introduced in the mass spectrometer by direct infusion and the optimal tuning conditions for both compounds in ES$^+$ (electrospray positive) mode were determined (Table 1). The most abundant species for both compounds were ammonium adducts, [M+NH$_4$]$^+$, m/z 566.6>369.4 for GlcChol and m/z 572.6>369.4 for $^{13}$C$_6$-GlcChol (see also Figure 1B). The product ion represents the cholesterol part of the molecule after loss of the glucose moiety. Because the $^{13}$C isotopes are on the glucose molecule, the daughter fragment of $^{13}$C$_6$-GlcChol has the same m/z ratio of 369.4.

Confirmation of compounds with m/z 566.6>369.4 being GlcChol was performed by demonstration of complete digestion to cholesterol using excess rGBA at pH 5.2 (McIlvaine buffer). The release of glucose was confirmed by a glucose oxidase assay.

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<th>Table 1. MS/MS instrument parameters.</th>
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Collection of NPC and GD patient plasma EDTA plasma (15 males and 3 females) were collected prior to therapy from Dutch patients suffering from type 1 GD, known by referral to the Academic Medical Center. Diagnosis of GD in patients was confirmed by genotyping and demonstration of deficient glucocerebrosidase activity in leukocytes or fibroblasts. Plasma samples were stored frozen at –20°C until further use. EDTA plasma samples of 42 male and 47 female control subjects were collected at the Academic Medical Center. The EDTA plasma samples of 15 NPC patients and 9 NPC carriers were collected at the Unidad de Investigación Traslacional in Zaragoza, Spain.

The status of affected or carrier of NPC disease was determined after the exomic sequencing of NPC1 and NPC2 genes, according to the presence of two or one mutations, respectively. Filipin stainings of fibroblasts were conducted to complete the diagnosis study. Plasma samples were stored frozen at -20 °C until further use. Approval had been obtained from the institutional ethics committee and informed consent according to the Declaration of Helsinki.

Quantification of total GlcChol in human plasma For quantitative analysis of GlcChol in samples of plasma, we developed a LC-MS/MS method using the MRM (multiple reaction monitoring) mode of the transitions mentioned above. Firstly, GlcChol was extracted from plasma from a healthy individual according the method of Bligh and Dyer (34) with a few modifications. 20 μL of plasma was pipetted in an Eppendorf tube (2 mL) and 20 μL of an internal standard solution, containing 0.1 pmol/μL of 13C6-GlcChol in methanol, was added, followed by addition of 280 μL methanol and 150 μL of chloroform. After brief mixing, the sample was left at room temperature for 30 min, mixed occasionally and centrifuged for 10 min at 15,700xg to spin down precipitated protein. The supernatant was transferred to an Eppendorf tube and 150 μL chloroform and 250 μL water were added to induce separation of phases. After centrifugation (5 min at 15,700xg) the lower, hydrophobic phase was transferred to a clean Eppendorf tube and the upper phase was washed by addition of 300 μL of chloroform. Lower phases were pooled and taken to dryness at 35°C under a nitrogen stream. Next, the residue was dissolved in 700 μL of butanol and 700 μL of water, mixed well and centrifuged for 10 min at 15,700xg. The upper phase (butanol) was transferred to a 1 mL tube with screw cap and the sample was dried under a gentle stream of nitrogen at 35°C. Subsequently, the residue was dissolved in 150 μL of eluent B by mixing and sonication and after centrifugation (5 min at 15,700xg) an aliquot of 100 μL was transferred into an UPLC vial with insert. 10 μL of the solution was injected for analysis.

Secondly, for the quantification of GlcChol in plasma, the sample was spiked with GlcChol (concentrations: 0-2.5-5-10-25-50-100-200-1000 pmol GlcChol/mL of plasma), internal standard was added and samples were extracted. The ratio, the area from transition GlcChol over the area from the transition 13C6-GlcChol, was plotted against the concentration of GlcChol spiked in the plasma samples. A linear response was obtained over the entire concentration range (y =0.0108x+1.9188, R² = 0.998). The within run variation (164.2 ± 4.3 pmol/mL with CV [coefficient of variation] % 2.6) and between run variation (166.8 ± 3.6 pmol/mL with CV% 2.2), was determined in plasma of a healthy volunteer by ten repetitive measurements.

The limit of detection (LOQ) was 0.5 pmol/mL plasma with a signal to noise ratio of three and the limit of quantitation (LOQ) was 0.9 pmol/mL plasma with a signal to noise ratio of 10. Calculation of the signal to noise ratio was done using the peak-to-peak method.

Analysis of GlcChol in animal tissues by LC-MS/MS 3 pmol of 13C6-labelled GlcChol in methanol was added to 30 μL of mouse tissue homogenate. Next, lipids were extracted according to the method of Bligh and Dyer (34) and GlcChol was analyzed by LC-MS as described above.

Analysis of GlcChol in COS-7 cells by LC-MS/MS COS-7 cells overexpressing GBA2 or GCS were homogenized by sonication on ice. Prior to extraction, 2 pmol of 13C6-labelled GlcChol in methanol (used as an internal standard) was added to 180 μL of homogenate. Next, lipids were extracted according to the method of Bligh and Dyer (34) by addition of methanol, chloroform and water (1:1:0.9, v/v/v) and the lower phase was taken to dryness under a stream of nitrogen. Isolated lipids were purified by water/butanol extraction (1:1, v/v) and GlcChol was analyzed by LC-MS as described above.
**Analysis of GlcCer and ceramide (Cer) in COS-7 cells by HPLC**

COS-7 cells overexpressing GBA2 or GCS, were homogenized by sonication on ice. Prior to extraction, 1 nmol of C17-sphinganine in methanol (used as an internal standard) was added to 100 µL of homogenate. Next, lipids were extracted according to the method of Bligh and Dyer (34) by addition of methanol, chloroform and water (1:1:0.9, v/v/v) and the lower phase taken to dryness under a stream of nitrogen. Isolated lipids were deacylated in a microwave oven, derivatized and analyzed by HPLC as described before (36).

**Analysis of GlcChol in RAW264.7 cells by LC-MS/MS**

3 pmol of 13C6-labelled GlcChol in methanol was added to 100 µL of RAW264.7 cell lysate. Next, lipids were extracted according to the method of Bligh and Dyer (34) and GlcChol was analyzed by LC-MS as described above.

**Protein concentration**

Determined using the Pierce BCA Protein Assay kit (Thermo Scientific) by the microplate procedure. Absorbance measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.) at 550nm.

**Statistical Analysis**

Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student’s t-test or Mann-Whitney U test. P values < 0.05 were considered significant. * P < 0.05, ** P < 0.01 and *** P < 0.001.

**Results**

**Quantification of GlcChol by LC-MS/MS**

To establish whether glucosyl-β-D-cholesterol (GlcChol) physiologically occurs in mammals, we firstly developed a LC-MS/MS procedure for its quantitative detection. For this purpose, a 13C6-isotope labelled GlcChol was synthesized (13C6-GlcChol). The use of the isotope labelled compound as internal standard avoids the need for corrections for extraction efficiency, chromatographic behavior and ionization efficiency, during quantification of GlcChol. To prevent undesired adduct formation, lipids were extracted in the absence of additional salts. To stimulate formation of desired ammonium adduct we incorporated 10 mM ammonium in the eluent.

Sensitive quantitative measurement of GlcChol proved feasible with 13C6-isotope labelled GlcChol as internal standard as shown in Figure 1A-C. The limit of detection (LOD) was 0.5 pmol/mL plasma, with a signal to noise ratio of 3 and a limit of quantitation (LOQ) of 0.9 pmol/mL plasma with a signal to noise ratio of 10. GlcChol was found to be an excellent substrate for recombinant GBA (rGBA Cerezyme®), even at sub-optimal conditions (absence of Triton X-100 and sodium taurocholate) (Figure 1D).
Analysis of GlcCer and ceramide (Cer) in COS-7 cells by HPLC

COS-7 cells overexpressing GBA2 or GCS, were homogenized by sonication on ice. Prior to extraction, 1 nmol of C17-sphinganine in methanol (used as an internal standard) was added to 100 µL of homogenate. Next, lipids were extracted according to the method of Bligh and Dyer (34) by addition of methanol, chloroform and water (1:1:0.9, v/v/v) and the lower phase taken to dryness under a stream of nitrogen. Isolated lipids were deacylated in a microwave oven, derivatized and analyzed by HPLC as described before (36).

Analysis of GlcChol in RAW264.7 cells by LC-MS/MS

3 pmol of 13C6-labelled GlcChol in methanol was added to 100 µL of RAW264.7 cell lysate. Next, lipids were extracted according to the method of Bligh and Dyer (34) and GlcChol was analyzed by LC-MS as described above.

Protein concentration

Determined using the Pierce BCA Protein Assay kit (Thermo Scientific) by the microplate procedure. Absorbance measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.) at 550nm.

Statistical Analysis

Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student’s t-test or Mann-Whitney U test. *P < 0.05, **P < 0.01 and ***P < 0.001.

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![Image of Figure 1. Quantification of GlcChol by LC-MS/MS.](image)

Demonstration of natural occurrence of GlcChol in mice

Next, we examined various tissues of wild-type (wt) mice on the presence of GlcChol. Relative high amounts of glucosylated sterol were noted for thymus, sciatic nerve, brain and lungs (see Figure 2A). The identity of the quantified structure (m/z 566.6) in thymus (and other tissues) was confirmed by its digestion by rGBA, showing that in wt animals more than 90% of the lipid measured is indeed glucosyl-β-D-cholesterol. However, in sciatic nerve and brain a significant proportion of m/z 566.6 was not digested by rGBA, suggesting that it may represent another glycosylated cholesterol, for example galactosyl-β-D-cholesterol.

The concentration of GlcChol in liver and thymus was determined for tissues of wt mice, animals lacking GBA2 (22) and LIMP-2 KO mice with markedly reduced GBA due to impaired transport to lysosomes (31). As shown in Figure 2B, C and D, the GlcChol concentration was markedly lower in all the tissues of GBA2-deficient animals analyzed compared to wt controls, especially in thymus. In contrast, in the GBA-deficient LIMP-2 KO mice no reduction in GlcChol, but
rather a significant increase in liver and plasma levels was observed (Figure 2C and D; \( P < 0.001 \) and \( P < 0.05 \), respectively). Mice with an induced GBA deficiency in the white blood cell lineage showed upon treatment with glucosylceramide synthase (GCS) inhibitor Genz-112638 (Eliglustat tartrate, Genzyme) partial reduction in plasma GlcCer (37) and an almost statistically significant reduction of elevated plasma GlcChol (Figure 2E, \( P = 0.07 \)). An increase in GlcChol was also observed in liver (Figure 2F, \( P < 0.001 \)), spleen and bone marrow (see Supplemental Figure 1) of mice with induced Gaucher disease. Correction of GBA deficiency by lentiviral gene therapy under the control of different promoters led in all cases to a concomitant reduction of GlcChol in these tissues (Figure 2F).

Figure 2. GlcChol in tissues of wt, GBA-deficient and GBA2-deficient mice. A. GlcChol (picomoles per milligram protein) in various tissues of wt male mice at 2 months of age (\( n = 3 \), mean ± S.D.). *Significant proportion of m/z 566.6 was not digestible by rGBA. B. GlcChol (nanomoles per gram wet weight) in thymus of wt, \( \text{Gba}^{-/-} \) and \( \text{Limp}^{-/-} \) male mice at 2 months of age (\( n = 3 \), mean ± S.D.). C. GlcChol (nanomoles per gram wet weight) in liver of wt, \( \text{Gba}^{-/-} \) and \( \text{Limp}^{-/-} \) male mice at 2 months of age (\( n = 3 \), mean ± S.D.). D. GlcChol (nanomolar) in plasma of wt, \( \text{Gba}^{-/-} \) and \( \text{Limp}^{-/-} \) male mice at 2 months of age (\( n = 3 \), mean ± S.D.). E. Plasma GlcChol (nanomolar) in wt (\( n = 27 \)), type 1 GD mice untreated (\( n = 40 \)) and treated with Genz-112638 (\( n = 27 \)) (mean ± S.D.). F. Liver GlcChol (nanomoles per gram wet weight) in wt mice (\( n = 6 \), mean ± S.D.), type 1 GD induced mice untreated (\( n = 6 \)), type 1 GD treated with lentiviral GBA cDNA gene therapy with macrophage specific promoter (CD68) (\( n = 8 \)), ubiquitously expressed human phosphoglycerate kinase (PGK) promotor (\( n = 12 \)) or gammaretroviral vector with the viral promoter spleen focus forming virus (SFFV) promotor (\( n = 8 \)). Data were analyzed using an unpaired t-test. * \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \).
**In vitro transglycosylation by β-glucosidases**

The enzymes GBA and GBA2 were both found able to hydrolyze GlcChol at conditions optimal for degradation of 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) (Supplemental Table 1).

The findings on GlcChol levels in wt, GBA-deficient and GBA2-deficient mice prompted us to study the ability of the three β-glucosidases GBA, GBA2 and GBA3 to generate glucosylated cholesterol by transglycosylation. We first studied this ability *in vitro* and reproduced the assay of Akiyama and co-workers (11) using 25-NBD-cholesterol as acceptor and detection of 25-NBD-glucosyl-cholesterol formation by TLC and fluorescence scanning. As source of enzyme we used rGBA and individually overexpressed GBA2, GBA3 and GCS in COS-7 cells. Overexpression of enzymes was checked by measuring enzymatic activity with appropriate assays (not shown).

Recombinant enzyme and COS-7 cell lysates were incubated with natural GlcCer (C18:1-GlcCer) as donor and 25-NBD-cholesterol as acceptor. Following incubation at optimal conditions for each enzyme with inclusion of UDP-Glc for GCS (see Methods section), lipids were extracted and subjected to HPTLC. As shown in Figure 3A, rGBA and cell lysates with overexpressed GBA2 generated an additional fluorescent lipid coinciding with 25-NBD-cholesterol-glucoside (Figure 3A). Inhibition of GBA with conduritol B epoxide (CBE) or GBA2 with AMP-DNM prevented formation of 25-NBD-cholesterol-glucoside (Figure 3A). Transglycosylation was hardly observed for cell lysates with overexpressed GBA3 and GCS (Figure 3A), recapitulating the findings by Akiyama and co-workers concerning GCS (10). We repeated the experiment using natural cholesterol as acceptor and determined the levels of formed GlcChol by LC-MS (Figure 3B). Again, GlcChol formation occurred in the presence of GBA (inhibitable by CBE) and in cell lysates with high GBA2 (inhibitable by AMP-DNM) (Figure 3B). Lysates of cells overexpressing GBA3 and GCS showed no additional GlcChol formation (Supplemental Figure 2). The pH optimum of GBA and GBA2 to generate GlcChol was next determined (see Supplemental Figure 3). In the case of GBA optimal activity was seen between pH 4.5-5.5 and for GBA2 between pH 6.0-7.0. We investigated with the same assay whether GlcSph, GalCer and GalSph also act as glycose donors in the transglycosylation catalyzed by GBA or GBA2 (see Supplemental Figure 3). This was not observed.

We next studied whether natural GlcChol (100 µM) can also act as donor in transglycosylation by incubating rGBA and lysates of cells overexpressing GBA2 or GBA3 in the presence of NBD-ceramide (40 µM) as acceptor. Lysates of cells overexpressing GBA2 and rGBA showed formation of fluorescent NBD-GlcCer (Figure 3C). This was not observed for lysates with overexpressed GBA3 (Figure 3C). Transglycosylation by both GBA and GBA2 occurs as an equilibrium reaction in which the glucose moiety is reversibly exchanged between cholesterol and ceramide.

We investigated in more detail potential transglycosylase activity of human GBA3 overexpressed in CHO-K1 cells. Overexpression of GBA3 increased β-glucosidase activity (Figure 3D). As shown in Figure 3E, overexpression of GBA3 did not affect transglycosylase activity from natural GlcCer (C16:0-GlcCer) to 25-NBD-cholesterol despite the long reaction time (20 h). Almost same results were observed in cell lysates incubated with natural GlcCer (C18:0-GlcCer or C24:1-GlcCer) as donor (data not shown).

Molecular docking of GlcChol in the GBA crystal structure was performed (see Supplemental Figure 4). The ligand GlcChol was built and regularized with ligand and superimposed on the bicyclic nojirimycin analogue ligand that was crystallized in complex with GBA (pdb [protein data bank] code 2XWE) using the program coot (see Supplemental Methods). GlcChol was found to be positioned such that the glucosidic bond is accessible to the catalytic residues Glu235 and Glu340. The cholesterol moiety concomitantly interacts with aromatic side chains.
Figure 3. *In vitro* transglucosylation of 25-NBD GlcChol by GBA and GBA2. A: rGBA and lysates of cells with overexpression of GBA2, GBA3 and GCS were incubated for 0 and 1 h with 25-NBD-cholesterol in the presence of C18:1-GlcCer as donor, and in the absence or presence of the respective specific β-glucosidase inhibitors: CBE (GBA), AMP-DNM (GBA2) and anDIX (GBA3) (26). Formation of 25-NBD-GlcChol was detected by HPTLC and fluorescence scanning. B: rGBA (in absence or presence of CBE) and lysates of cells with overexpression of GBA2 (in presence of CBE and in absence or presence of AMP-DNM) were incubated for 0 and 1 h with cholesterol in the presence of C18:1-GlcCer as donor. Formation of GlcChol was detected by LC-MS. The percentage of inhibition of GlcChol formation by the respective inhibitors is shown. Data presented as mean ± S.D.. C: rGBA and lysates of cells with overexpression of GBA2 and GBA3 were incubated for 0 and 1 h with GlcChol in the presence of NBD-Cer. Formation of NBD-GlcChol was detected by HPTLC and fluorescence scanning. D: β-glucosidase activity in lysates of CHO-K1 cells overexpressing GBA3. The activity was measured in the absence or presence of the specific β-glucosidase inhibitors: 0.5 mM CBE and/or 0.3 mM NB-DGJ. CBE and NB-DGJ sensitive activities were defined as activities derived from GBA (black box) and GBA2 (gray box), respectively. CBE and NB-DGJ insensitive activity was defined as activity derived from GBA3 (dotted box). Mock represent the cells transfected with empty vector. E: Transglucosylase activity in lysates of cells with overexpression of GBA3. Data (n = 3, mean ± S.D.) were analyzed using an unpaired t-test. *P < 0.05 and **P < 0.01. Black, gray and dotted lines showed the significant difference of the activity derived from GBA, GBA2 and GBA3, respectively.
Metabolism of GlcChol in cultured COS-7 cells

Next we studied factors influencing the formation of GlcChol content in cultured green monkey kidney COS-7 cells. We firstly studied the impact of overexpressed GBA2 and GCS. Figure 4 shows the effect on cellular GlcChol and GlcCer levels. Only overexpression of GCS led to increased levels of GlcCer (Figure 4A). GlcChol was not changed by overexpression of GBA2, but overexpression of GCS caused a twelve-fold increase in this lipid. Importantly, inhibition of GBA2 activity with low nanomolar AMP-DNM (38) resulted in reduced cellular GlcChol. Even in cells with overexpressed GCS the elevation in GlcChol was prevented (Figure 4B). In contrast, inhibition of GBA with CBE hardly diminished increased GlcChol level in cells with overexpressed GCS. These findings suggest that GCS does not generate GlcChol itself, but is required to generate sufficient GlcCer to be used as donor in formation of GlcChol by transglucosylation. This transglucosylation in COS-7 cells is particularly mediated by GBA2, and not GBA. The latter notion is consistent with the finding that GBA2 deficiency in mice, and not that of GBA, is accompanied with reduced GlcChol levels of tissues.

Figure 4. GlcChol in COS-7 cells manipulated in GSL metabolizing enzymes. A: GlcCer (nanomoles per milligram protein) in COS-7 cells without overexpression of enzymes (control), overexpressed GBA2 and GCS. Cells were incubated for 2 days with indicated inhibitors of GBA2 (AMP-DNM) and GBA (CBE). B: GlcChol (picomoles per milligram protein) in same cells. Data (n = 4, mean ± S.D.) were analyzed using an unpaired t-test. *** P < 0.001.

GlcChol in Niemann-Pick disease type C mice and U18666A treated cells

In Niemann Pick disease type C (NPC) disease, cholesterol accumulates prominently in lysosomes as the result of impaired export from the compartment due to defects in either Npc1 or Npc2. In liver of spontaneous Npc1<sup>nih/nih</sup> (29, 39, 40) and Npc1<sup>spm/spm</sup> mice (30, 41) we observed a remarkable, 25-fold, increase in GlcChol content (Figure 5A and B). The identity of the measured glucosylated sterol was examined by digestion with rGBA. While more than 90% of the GlcChol in liver of wt mice was digested to cholesterol, in the case of material from NPC mice this was only around 70% (not shown). Based on this finding, it seems likely that part of the elevated compound with m/z 566.6>369.4 in NPC liver consists of cholesterol molecules modified differently with sugar, indistinguishable from glucosyl-β-D-cholesterol with the LC-MS method.
To experimentally substantiate the observations made for GlcChol in liver of NPC mice, we induced impaired cholesterol export from lysosomes in murine macrophage RAW264.7 cells by exposure to U18666A (42). Following lysosomal cholesterol accumulation, cells showed elevated GlcChol. Concomitant inhibition of lysosomal GBA by CBE prevented the increase of GlcChol in U18666A exposed cells (Figure 5C). Formation of excessive GlcChol was also prevented by the presence of 1 mM β-methyl-cyclodextrin, an agent known to reduce intralysosomal cholesterol in NPC cells (43). This indicates that during extreme intralysosomal accumulation of cholesterol, GBA actively generates GlcChol. In normal lysosomes GBA most likely largely degrades the glucosylated sterol.

Figure 5. GlcChol abnormalities in NPC. A: GlcChol (nanomoles per gram wet weight) in liver of BALB/c Npc1+/+ , Npc1+/− and Npc1−/− male mice at 80 days of age (n = 3, mean ± S.D.) B: GlcChol (nanomoles per gram wet weight) in liver of C57BLKS Npc1+/pim and Npc1−/pim male mice at 80 days of age (n = 3-5, mean ± S.D.). C: GlcChol (picomoles per milligram protein) in RAW264.7 cells incubated with indicated concentration U18666A for 1 day in absence and presence of CBE inhibiting GBA (n = 3 mean ± S.D.). D: GlcChol (picomoles per milligram protein) in RAW264.7 cells incubated with 10 µM U18666A for 8 h and in subsequent absence or presence of 1 mM β-methyl-cyclodextrin (β-mCD) reducing intralysosomal cholesterol for 18 h (n = 3 mean ± S.D.). Data were analyzed using an unpaired t-test. ** P < 0.01 and *** P < 0.001.

GlcChol in NPC and GD patients

Finally, we determined GlcChol levels in plasma of untreated symptomatic type 1 GD patients as well as in NPC patients, carriers and healthy controls. As shown in Figure 6A, GlcChol tends to be increased in plasma of symptomatic GD patients and less prominently in that of NPC patients. The abnormalities in GD patients are more pronounced when plasma GlcChol is related to cholesterol (Chol, Figure 6B). Investigation of plasma specimens of type 1 GD patients treated with GCS inhibitor Eliglustat (Figure 6C), showed a prominent reduction upon inhibition of GSL synthesis. Treatment of matched patients with the weaker GCS inhibitor Miglustat (Zavesca®, Actelion) also led to a reduction of GlcChol, albeit slower (Figure 6C). Of note, treatment of matched patients with rGBA Cerezyme (enzyme replacement therapy; ERT) did not reduce GlcChol to the same extent, despite impressive clinical improvements in these patients (Figure 6C) (B.E. Smid et al. unpublished).
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Figure 5. GlcChol abnormalities in NPC.

A: GlcChol (nanomoles per gram wet weight) in liver of BALB/c Npc1 +/+ , Npc1 +/nih and Npc1 nih/nih male mice at 80 days of age (n = 3, mean ± S.D.). B: GlcChol (nanomoles per gram wet weight) in liver of C57BLKS Npc1 +/spm and Npc1 spm/spm male mice at 80 days of age (n = 3-5, mean ± S.D.). C: GlcChol (picomoles per milligram protein) in RAW264.7 cells incubated with indicated concentration U18666A for 1 day in absence and presence of CBE inhibiting GBA (n = 3 mean ± S.D.). D: GlcChol (picomoles per milligram protein) in RAW264.7 cells incubated with 10 µM U18666A for 8 h and in subsequent absence or presence of 1 mM β-methyl-cyclodextrin (β-mCD) reducing intralysosomal cholesterol for 18 h (n = 3 mean ± S.D.). Data were analyzed using an unpaired t-test. ** P < 0.01 and *** P < 0.001.

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Figure 6. Plasma GlcChol in LSD patients and healthy individuals. A: Plasma GlcChol (nanomolar) in healthy individuals, type 1 GD patients, NPC carriers and NPC patients. B: Ratio of GlcChol/cholesterol (Chol) in plasma of healthy individuals, type 1 GD patients, NPC carriers and NPC patients. C: Percentage reduction in plasma GlcChol of matched type 1 GD patients following Eliglustat, Miglustat and ERT treatment compared to pre-treatment values. Data were analyzed using the Mann-Whitney U test. ** P < 0.01 and *** P < 0.001.

Discussion

Recently, Akiyama and colleagues (11) demonstrated that GBA, either pure recombinant protein or enzyme in fibroblast lysates, can generate GlcChol by transglucosylation of cholesterol when provided with GlcCer as donor. The physiological significance of glucosylation of cholesterol is hypothetically great since it renders a molecule far more water soluble. To establish the natural occurrence of GlcChol in mammals, we first needed to develop a quantitative and sensitive assay for quantification of GlcChol in plasma, cells and tissues. The method developed by us makes use of newly synthesized 13C6-GlcChol as internal standard. Regarding extraction efficiency, chromatographic behavior and ionization characteristics, the natural and isotope labelled compounds are identical, so no correction for the sample matrix (ion suppression or ion enhancement) is required.

With sensitive quantification of GlcChol in place, we next observed that almost all tissues of mice show GlcChol. The relative high amount of the glucosylated sterol in the thymus, several
nanomoles per gram wet weight, is striking and deserves special attention in view of noted abnormalities in NKT and B-cells in GBA-deficient GD patients (44). It has been speculated by Mistry and colleagues that elevated GlcCer or GlcSph via binding to CD1 may be causing this phenomenon (44). It will be now of interest to study whether GlcChol interacts with CD1 since abnormalities in concentration of this lipid in GBA-deficient GD patients are likely.

Indeed, we recapitulated the finding that GBA is able to form GlcChol by transglucosylation of cholesterol, at least in vitro. Artificial β-glucosidases like 4MU-Glc may in vitro act as donor in this reaction as well as natural GlcCer. GlcChol is on the other hand also an excellent substrate for hydrolysis by GBA. Our findings suggest that GBA normally lowers GlcChol levels. GBA-deficient LIMP2 KO mice show modestly elevated GlcChol in several tissues and the same is observed in GBA\textsuperscript{null/flox} mice with induced type 1 GD (32). Finally, plasma GlcChol is elevated in symptomatic type 1 GD patients. The actual role played by GBA in GlcChol metabolism, degradation versus synthesis, might be highly dependent on local concentrations of donors (GlcCer and GlcChol) and acceptors (ceramide and cholesterol) in the transglucosylation equilibrium of the enzyme. The importance of this is suggested by some observations made in the course of our investigation. In the first place, high intralysosomal cholesterol concentrations appear to favor formation of GlcChol by GBA. This is indicated by the 25-fold elevated GlcChol in liver of two different models of NPC disease in mice. In accordance with this, induction of lysosomal cholesterol accumulation with U18666A in cells causes a rapid increase in GlcChol, which was noted to be abolished by inactivation of GBA. Consequently, under normal conditions, GBA seems to promote GlcChol degradation, but under excess cholesterol accumulation in lysosomal membrane, such found in NPC, the equilibrium of the metabolism is altered to favor GlcChol formation by GBA.

We observed that the non-lysosomal β-glucosidase GBA2 can also generate in vitro GlcChol through transglucosylation. Again, GlcCer was found to be an excellent donor for this reaction. GBA2 is equally able to degrade GlcChol. Our finding of reduced GlcChol levels in GBA2 KO mice suggests, but does not entirely prove, that GBA2 in vivo contributes to the presence of GlcChol. The enzyme GBA2 is located differently in cells from lysosomal GBA, with its catalytic pocket exposed to cytoplasmic leaflet of membranes. It likely encounters different concentrations of GlcCer and cholesterol than GBA given its location close to the cellular sites of de novo synthesis of these lipids. To maximally form GlcChol through transglucosylation, high concentrations of GlcCer as donor and high concentrations of cholesterol as acceptor are optimal. Vice versa, low concentrations of GlcCer and cholesterol will reduce net GlcChol formation. This consideration holds equally for GBA2 and GBA. Fluctuations in sterols and sphingolipids conceivably occur in cells, for example after uptake of cholesterol-rich lipoproteins or upon release of ceramide from sphingomyelin. The ability to maintain some equilibrium between (glucosylated) sphingolipids and sterols by transglucosylating β-glucosidases may have beneficial buffering effects for cells. Of interest in this respect is our finding that inhibition of GCS leads to reduction of GlcChol in cultured cells, plasma of mice and plasma of GD patients. This strongly suggests that the availability of GlcCer is an important driver of formation of GlcChol through transglucosylation. Since the β-glucosidases GBA and GBA2 also hydrolyze GlcCer, and thus tend to lower its concentration, the exquisite balance of various GlcCer metabolizing enzymes and local cholesterol concentrations will determine GlcChol formation in subcellular compartments.

GlcChol is far more water soluble than cholesterol and therefore more suited for transport. The relatively low steady concentration of GlcChol does not exclude a vital role as intermediate in a transport pathway. Tentatively, water soluble GlcChol formed by transglucosylation at one cellular site would be transported and reconverted at the destination site back to more inert cholesterol. The combination of the two enzymes GBA and GBA2 could provide of such a mechanism, without need for ATP. Of interest in view of this speculation is that LIMP2, the membrane protein interacting with
GBA, is recently shown to have a cholesterol binding site and potentially even a tunnel/channel function (45).

A final consideration concerns the possible pathophysiological consequences of disturbed GlcChol metabolism. It seems likely that in individuals with abnormal GlcCer metabolism, as for example GBA-deficient GD patients, secondary abnormalities in GlcChol occur. Future research will need to reveal whether such abnormalities in GlcChol or in other glucosylated metabolites contribute to particular symptoms associated with GD. In conclusion, GlcCer has earlier been identified as an important structural membrane component and intermediate in GSL biosynthesis. In addition, it is known to act as important sink for pro-apoptotic ceramide (46, 47). Our study suggests that GlcCer may furthermore act as glucosyl-donor in the formation of GlcChol via transglycosylation.

Acknowledgements
We thank colleagues in the Academic Medical Center SPHINX clinic, Bouwien Smid and Carla Hollak, for sharing Gaucher disease patient materials. The longstanding support by Dutch Gaucher disease patients is acknowledged.

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Disclosures
None relevant to this study.

References


Glucosylated cholesterol metabolism by β-glucosidases

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Supplemental Information

Supplemental Methods

Reagents C18-GlcSph (D-glucosyl-β1-1′-D-erythro-sphingosine), C18-GalCer (D-galactosyl-β1-1′ N-palmitoyl-D-erythro-sphingosine) and GalSph (D-galactosyl-β1-1′-D-erythro-sphingosine) were obtained from Avanti Polar Lipids (Alabaster, USA).

Molecular Modeling The ligand GlcChol was build and regularized with ligand (1) and superimposed on the bicyclic nojirimycin analogue ligand that was crystallized in complex with GBA (pdb code 2XWE) (2) using the program coot (3). Supplementary Figure 4 shows the resulting model of GBA complexed with GlcChol.

Supplemental References


Supplemental Results

Supplemental Table 1. Degradation of GlcChol by GBA and GBA2.

<table>
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<tr>
<th>Input: nmol 4MU-β-Glc hydrolysis per mL/min</th>
<th>Percentage GlcChol digestion (200 pmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rGBA</td>
<td>1000</td>
</tr>
<tr>
<td>GBA2</td>
<td>0.04</td>
</tr>
</tbody>
</table>
**Supplemental Figure 1. Increased GlcChol in spleen and bone marrow of mice with induced type 1 GD.**

A. Spleen and B. bone marrow GlcChol in wt mice, type 1 GD induced mice untreated, type 1 GD treated with lentiviral GBA cDNA gene therapy with macrophage specific promoter (CD68), ubiquitously expressed human phosphoglycerate kinase (PGK) promoter or gammaretroviral vector with the viral promoter spleen focus forming virus (SFFV) promoter. Data were analyzed using an unpaired t-test. * P < 0.05, ** P < 0.01 and *** P < 0.001.

**Supplemental Figure 2. In vitro formation of GlcChol by different β-glucosidases.**

Recombinant rGBA and lysates of cells with overexpression of GBA2, GBA3 or GCS were incubated for 0 and 1 h with cholesterol in the presence of C18:1-GlcCer as donor. Formation of GlcChol (nmol/L*h) was detected by LC-MS. Inhibition of GlcChol formation by the respective β-glucosidase inhibitors – CBE (GBA), AMP-DNM (GBA2) and anDIX (GBA3) – is shown.
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Supplemental Figure 3. In vitro formation of GlcChol: pH dependence and donor preference.

A. rGBA (in McIlvaine buffer 0.15 M, 0.2% taurocholate and 0.1% Triton X-100) and lysates of cells overexpressing GBA2 (in McIlvaine buffer 0.15 M) were incubated for 1 h with 25-NBD-cholesterol in the presence of C18:1-GlcCer as donor at different pHs. B. rGBA (in McIlvaine buffer 0.15M pH 5.2, 0.2% taurocholate and 0.1% Triton X-100) and lysates of cells overexpressing GBA2 (in McIlvaine buffer 0.15 M pH 5.8) were incubated for 1 h with 25-NBD-cholesterol in the presence of different donors (100 μM): 4-MU-glucopyranoside, C18:1-GlcCer, C18-GlcSph, C18-GaICer and C18-GalSph.
Supplemental Figure 4. Molecular docking of GlcChol in GBA crystal structure 2XWE.
GlcChol docked on GBA crystal structure 2XWE. GlcChol is shown in green with its oxygen atoms in red. GBA is shown in blue (and gold) with the catalytic residues Glu235 and Glu340 labeled. Side chains that are within 5 Å of GlcChol are also displayed.