Chapter 15

β-Xylosidase and transxylosylase activities of human glucocerebrosidase

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β-Xylosidase and transxylosidase activities of human glucocerebrosidase

Mina Mirzaian[1*], Maria J. Ferraz[1*], Daphne E.C. Boer[1], Saskia V. Oussoren[1], Marc Hazeu[1], Sybrin P. Schröder[3], Karen Ghauharali[2], Jasper Wermink[1], Per Haberkant[1,b], Edward Blommaart[2], Roelof Ottenhoff[2], André R.A. Marques[1,a], Rianne Meijer[1], Wouter Kallemeijn[1], Rolf G. Boot[1], Herman S. Overkleeft[3], Navraj S. Pannu[4], Johannes M. Aerts[1#].

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[1] Department of Medical Biochemistry, Leiden Institute of Chemistry (LIC), Leiden University, The Netherlands;

[2] Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands;

[3] Department of Bio-organic Synthesis, LIC, Leiden University, The Netherlands;

[4] Department of Macromolecular Biochemistry, LIC, Leiden University, The Netherlands aPresent address: Department of Biochemistry, Christian-Albrechts-Universität, Kiel, Germany

[b] Present address: Cell Biology and Biophysics Unit EMBL, Heidelberg.

[*] These authors contributed equally to this work, and should be considered as first authors.

#Corresponding author:

Johannes M. F. G. Aerts
Department of Medical Biochemistry, room DE.1.19
Leiden Institute of Chemistry
Einsteinweg 55, 2300 RA Leiden, The Netherlands
Phone: +31 (0) 71 5274771
E-mail: j.m.f.g.aerts@lic.leidenuniv.nl

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Abbreviations: 25-NBD-cholesterol: 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl) methyl] amino]-27-norcholesterol; GlcChol: 1-O-cholesteryl-β-D-glucose; GlcCer: glucosylceramide; XylChol: 1-O-cholesteryl-β-D-xylose; 4-MU: 4-methylumbelliferone; LSD: lysosomal storage disease; GD: Gaucher disease; NPC: Niemann Pick Type C; GSL: glycosphingolipid.

Abstract

Glucocerebrosidase (GBA1) is a lysosomal retaining β-glucosidase that hydrolyzes β-glucosidic substrates and transglucosylates cholesterol to cholesterol-β-glucoside (GlcChol). Here we demonstrate that recombinant human GBA1 also cleaves 4-methylumbelliferyl-β-D-xylose (4-MU-β-Xyl), being stimulated in this activity by saposin C. GBA1 is furthermore shown to transxylosylate fluorescent 25-NBD-cholesterol and natural cholesterol using 4MU-β-Xyl as donor. Formed xylosyl-cholesterol (XylChol) acts as subsequent acceptor to render di-xylosyl-cholesterol. As GBA1, the cytosolic β-glucosidase GBA3 *in vitro* shows β-xylosidase and transxylosylase activities, but not the membrane-bound cellular β-glucosidase GBA2. When exposed to 4MU-β-Xyl, intact cells also generate xylosylated cholesterols, independent of the presence of glucosylceramide synthase. This synthesis is promoted by the drug U18666A causing lysosomal cholesterol accumulation. Prior inactivation of cellular GBA1 with conduritol-β-epoxide prevents formation of xylosylated cholesterols by cells. In conclusion, our findings point to further catalytic versatility of GBA1 and warrant examination of occurrence of xylosylated cholesterol as well as the existence of other xylosylated lipids.

Introduction

The aldopentose xylose mimics the six-membered cyclic pyranose glucose except for the lacking pendant CH_2OH group. As main building block of xylan, xylose is a major plant sugar [1]. In animals xylose is added by UDP-xylose dependent xylosyltransferases as the first saccharide to sidechain hydroxyls of serine or threonine residues during O-glycosylation of proteoglycans. This step is essential in synthesis of glycosaminoglycans like heparan sulfate, keratan sulfate and chondroitin sulfate [2]. The human body is unable to synthesize *xylose de novo*. UDP-xylose is however formed from UDP-glucuronate by UDP-glucuronic acid decarboxylase 1 encoded by the UXS1 gene [3]. Since the first investigations by Fisher & Kent and Patel & Tappel in the late sixties, degradation of β-xylosides in animals is thought to rely on β-glucosidases [4, 5]. This does not come as a surprise in view of the structural similarity of xylose with glucose. We earlier demonstrated that indeed the lysosomal acid β-glucosidase GBA1, also known as glucocerebrosidase, hydrolyzes 4-methylumbelliferyl-β-xyloside (4-MU-β-Xyl), in contrast to the non-lysosomal β-glucosidase GBA2 [6]. Inherited defects in GBA1 cause Gaucher disease (GD), a progressive disorder characterized by the accumulation of macrophages loaded with glucosylceramide (GlcCer) in tissues [7, 8]. No accumulation of β-D-xylose-containing glycopeptides in GD patients has been reported, but this possibility has not been actively studied. More recently another catalytic capacity of GBA1 has been recognized: the transfer of glucose from β-glucoside substrates to cholesterol, thus generating β-D-glucosylcholesterol (GlcChol) [9, 10]. Glew and colleagues earlier demonstrated that *in vitro* GBA1 can transfer glucose to retinol via transglucosylation [11]. Generation of GlcChol by GBA1 is not merely a test tube phenomenon, but also takes place *in vivo* [10]. In Niemann Pick type C disease (NPC), intralysosomal cholesterol is markedly increased due to genetic defects in any of the two proteins NPC1 or NPC2 mediating the egress of the sterol from lysosomes [12]. During this pathological condition, GBA1 actively generates GlcChol [10]. Formation of GlcChol can also be experimentally induced by incubating cells with U18666A, an inhibitor of efflux of cholesterol from lysosomes. The transglucosylation reaction in cells is prohibited by concomitant inhibition of GBA1 [10]. The β-glucosidase GBA2, tightly associated to the cytoplasmic leaflet of membranes, also exerts transglucosidase activity *in vitro* and *in vivo* [10, 13-15].

The earlier findings on glycon substrate specificity of GBA1 and the recently noted transglucosidase activity of the enzyme prompted us to examine whether GBA1 is also able to generate xylosyl-β-D-cholesterol (XylChol). We here report on the *in vitro* xylosylation of cholesterol by GBA1, rendering not only XylChol, but also di-xylosyl-cholesterol and even small amounts of tri-xylosyl-cholesterol (Xyl_2Chol and Xyl_3Chol, respectively). Our findings are discussed in view of physiological relevance and potential existence of additional xylosylated lipids.

Materials and Methods

Materials. 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (25-NBD-cholesterol) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 4-methylumbelliferyl β-D-glucose (4-MU-Glc) and 4-methylumbelliferyl β-D-xylose (4-MU-Xyl) were purchased from Glycosynth™ (Winwick Quay Warrington, Cheshire, England). Cholesterol trafficking inhibitor U18666A, 1-O-cholesteryl-β-D-glucose (β-cholesteryl glucose, β-GlcChol) and ammonium formate (LC-MS quality) were from Sigma-Aldrich (St Louis, MO, USA). GBA1 inhibitor Conduritol-β-epoxide (D, L-1,2-anhydro-*myo*-inositol; CBE) was from Enzo Life Sciences Inc. (Farmingdale, NY, USA), GBA1 inhibitor cyclophellitol, GBA2 inhibitor N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin (AMP-DNM) and GBA3 inhibitor α-1-C-nonyl-DIc (anDIX) were synthesized at

Leiden Institute of Chemistry (Leiden, The Netherlands) [16, 17]. Cerezyme®, a recombinant human GBA1 was obtained from Genzyme (Genzyme Nederland, Naarden, The Netherlands). LC-MS-grade methanol, 2-propanol, water, and HPLC-grade chloroform was purchased from Biosolve. D-xylo-cyclophellitol was synthesized as reported earlier [18].

Mouse materials. *Npc1*$^{-/-}$ mice (*Npc1*^{nih}), along with wild-type littermates (*Npc1*$^{+/+}$), were generated by crossing *Npc1*$^{+/-}$ males and females. The heterozygous BALB/c Nctr-*Npc1*^{m1N}/J mice (stock number 003092) were obtained from the Jackson Laboratory (Bar Harbor, USA). Mouse pups were genotyped according to published protocols [19]. 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 free 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). Animals were first anesthetized with a dose of Hypnorm (0.315 mg/mL fentanyl 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 and expression of cDNAs encoding β-glucosidases GBA2 and GBA3. Stable GBA2 expressing HEK293T cells were generated as follows. The PCR-amplified human GBA2 (GBA2, acc. nr: NM_020944.2) coding sequence (using the following oligonucleotides: sense. 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCACCATGGGGACCCAGGATCCAG-3' and antisense 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACTCTGGGCTCAGGTTTG-3') was cloned into pDNOR-221 and sub-cloned in pLenti6.3/TO/V5-DEST using the Gateway system (Invitrogen). Correctness of the construct was verified by sequencing. To produce lentiviral particles HEK293T cells were transfected with pLenti6.3-GBA2 in combination with the envelope and packaging plasmids pMD2G, pRRE and pRSV. Subsequently, culture supernatant containing viral particles was collected and used for infection of HEK293T cells. Selection using blasticidin for several weeks rendered cells stably expressing human GBA2 as determined by activity assays. For stable expression of human GBA3 in HEK293T cells, the PCR-amplified GBA3 (GBA3, acc. Nr: NM_020973.4) coding sequence (using the following oligonucleotides: sense. 5'-GAATTCGCCGCCACCATGGCTTTCCCTGCAGGATTTG-3' and antisense 5'-GCGGCCGCAGATGTGCTTCAAGGCCATTG-3') was cloned in pcDNA3.1/Zeo and transfected into HEK293T cells using FuGENE® 6 Transfection Reagent (Promega Benelux, Leiden, The Netherlands). Selection using Zeocin for several weeks rendered cells stably expressing human GBA3 as determined by activity assays.

***In vitro* assays with fluorogenic 4-methylumbelliferyl-β-D-glycosides.** Enzymatic activity of GBA1 was measured with 3.7 mM 4-MU-β-Glc or 4-MU-β-Xyl, dissolved in 150 mM McIlvaine buffer (pH 5.2 supplemented with 0.2 % (w/v) sodium taurocholate, 0.1 % (v/v) Triton X-100) and 0.1 % (w/v) BSA) [20]. The reaction was stopped with NaOH-glycine (pH 10.3), and fluorescence was measured with a fluorimeter LS55 (Perkin-Elmer, Beaconsfield, UK) at λ_{ex} 366 nm and λ_{em} 445 nm. Enzymatic activity of GBA2 was measured in lysates of cells overexpressing the enzyme using the same conditions as above but without the presence of detergents and at pH 5.8. Enzymatic activity of GBA3 was measured in the absence of detergents at pH 7.0 [17].

Stimulation of GBA1 activity by the activator protein saposin C, produced recombinantly in *E. coli* [21], was monitored with 3.7 mM 4-MU-β-Glc as substrate in 150 mM McIlvaine buffer pH 4.5 containing 0.1 % (w/v) BSA and 0.4 mg/mL phosphatidylserine [22].

***In vitro* assay of transglycosidase activity with fluorescent 25-NBD-Cholesterol as acceptor.** Lysates of HEK293 cells overexpressing GBA2, and GBA3, and recombinant GBA1 were used to determine transglycosidase activity of each enzyme. The assays were performed as described earlier [10]. First, 40 µL of cell homogenates overexpressing GBA2, or GBA3 were pre-incubated with 10 µL of 25 µM CBE in water for 20 min (samples containing diluted recombinant GBA1 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 4-MU-β-Xyl or 4-MU-β-Glc) and 40 µM of acceptor (25-NBD-cholesterol), was added. Transglycosidase activity of GBA2 overexpressing cells was measured in a 150 mM McIlvaine buffer pH 5.8 and the assay for recombinant GBA1 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 and appropriate inhibitors [10]. After 16h 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 [23]. Thereafter lipids were separated by thin layer chromatography 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) [6].

***In vitro* assay of transglycosidase activity with cholesterol as acceptor.** Assays with natural cholesterol as acceptor were performed exactly as described in the section above and the subsequent analysis of products was performed by LC-MS/MS as described in the section below. In brief, pure recombinant GBA1 was incubated at 37 ˚C with 32 µM cholesterol and 3.0 mM 4-MU-β-Xyl or 4-MU-β-Glc in 250 µl 150 mM McIlvaine buffer pH 5.2 containing 0.1% BSA, 0.1% Triton X-100 and 0.2% sodium taurocholate for the indicated time periods. The incubations were stopped by snap-freezing and lipids were extracted according to Bligh and Dyer.

Assays with cultured RAW264.7 and HEK293 cells. Experiments with cultured RAW264.7 and HEK293 cells exposed to 3.7 mM 4-MU-β-Xyl in the medium, either in the absence and presence of U18666A (10 µM), inducing lysosomal cholesterol accumulation, were performed as described earlier [10]. Lysosomal GBA1 was irreversibly inhibited by prior incubation of cells with 300 µM CBE. Cells were harvested and lipids extracted as earlier described [10]. Glucosylceramide synthase (GCS) in HEK293 cells was knocked-down by the CRISPR/Cas9 system [24].

Analysis of GlyChol and XylChol by LC-MS/MS. A Waters Xevo-TQS micro 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, Milford, MA, USA). Tuning conditions for both GlcChol and XylChol's in ES^+ (electrospray positive) mode are presented in Supplemental Table 1. GlcChol, $^{13}C_6$-GlcChol and XylChol's 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_2O 90:10 (v/v) containing 10 mM ammonium formate (Eluent A) and methanol containing 10 mM ammonium formate (Eluent B). The column temperature and the temperature of the auto sampler were kept at 23°C and 10°C respectively during the run. The flow rate was 0.25 mL/min. volume of injection 10 µL.

For the identification of a new compound of interest, the extracted sample was dried and dissolved in 100µL of Eluent B. The sample was introduced in the mass spectrometer using LC-MS (0 to 5.5 min to the detector). MS parents scan and daughters scan were performed (Figure 2). As for GlcChol [10], the most abundant species of XylChol are ammonium adducts, $[M+NH_4]^+$ and the product ion 369.3 represents the cholesterol part of the molecule after loss of the xylose moiety. Ammonium adducts of XylChol, Xyl_2Chol and Xyl_3Chol show the transitions 536.5>369.3, 668.5>369.3 and 800.5>369.3 respectively. The transitions for GlcChol are 566.5>369.3 and for $^{13}C_6$-GlcChol 572.5>369.3.
For Multiple Reaction Monitoring (MRM) the UPLC program was applied during 5.5 minutes consisting of 10% eluent A and 90% eluent B. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0 to 0.8 min) and after (4.5 to 5.5 min) the elution of the analytes to prevent system contamination. The retention time of both GlcChol and the internal standard $^{13}C_6$-GlcChol was 1.36 min. XylChol's were generated *in vitro* by incubation of GBA1 with cholesterol. The retention time of XylChol, Xyl_2Chol and Xyl_3Chol was 1.71 min, 1.49 min and 1.40 min respectively (Supplemental Figure 1).

LC-MS/MS quantitation of GlcChol and XylChol's produced *in vitro.* Following incubation of GBA1 and cholesterol with either 4-MU-β-Glc or 4-MU-β-Xyl, from 180 µL of sample, to which 12.5 pmol of $^{13}C_6$-GlcChol in methanol was added, lipids were extracted according to the method of Bligh and Dyer by addition of methanol, chloroform and water (1:1:0.9, v/v/v). The lower phase was taken to dryness in an Eppendorf concentrator. Isolated lipids were purified by water/butanol extraction (1:1, v/v). The upper phase (butanol phase) was taken to the dryness. The residue is dissolved in 100 µL eluent B, sonicated in bath sonicator and samples were analyzed by LC-MS.

LC-MS/MS quantitation of GlcChol and XylChol's in cultured cells. Cells were homogenized in water by sonication on ice. The protein concentration was around 1 mg/mL. Prior to extraction, 12.5 pmol of ^{13}C-labelled GlcChol in methanol (used as an internal standard) was added to 100 µL of homogenate. After protein precipitation, the supernatant was further treated as described above.

Protein determination. Protein was measured with the Pierce BCA Protein Assay kit (Thermo Scientific). Absorbance was measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.) at 562 nm.

Statistical Analysis. Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student's t-test. *P* values < 0.05 were considered significant. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

Cleavage of 4-methylumbelliferyl-β-D-xylose by GBA1.

We first compared the ability of pure recombinant hGBA1 to cleave 4-MU-β-Xyl and 4-MU-β-Glc. The enzyme releases fluorescent 4-MU from both substrates, but the noted activity towards 4-MU-β-Xyl is around 70-fold lower as the result of a higher Km and lower Vmax (Table 1). The activity of GBA1 towards both substrates shows a similar pH optimum (Figure 1A) and stimulation by taurocholate (Figure 1B). The stimulatory effect of recombinantly produced saposin C on GBA1-mediated cleavage of 4-MU-β-Glc and 4-MU-β-Xyl is comparable (Figure 1C). The $k_{cat}//K_m$ of recombinant GBA1 is about 40-fold higher for 4-MU-β-Glc than 4-MU-β-Xyl (Table 1). The retaining β-glucosidase GBA1 employs the double displacement mechanism for catalysis with E340 as nucleophile and E325 as acid/base [25]. Blocking glutamate E340 by covalent linkage of the suicide inhibitor cyclophellitol abolishes activity of GBA1 [26]. The activity of GBA1 towards 4-MU-β-Glc and 4-MU-β-Xyl substrates was found to be both inhibited by pre-incubation for 90 minutes followed by an activity assay with the substrates for 60 minutes. The slightly higher apparent IC_{50} observed with 4-MU-β-Glc (85 nM) than with 4-MU-β-Xyl (61 nM) is likely explained by the greater protection against irreversible inhibition of GBA1 by the β-D-glucose substrate. Recently a xylose analogue of cyclophellitol was synthesized [18]. It was observed that GBA1 is also irreversibly inactivated by D-xylo-cyclophellitol, although with lower affinity than cyclophellitol (Table 1). Again the apparent IC_{50} determined with 4-MU-β-Glc (10.16 μM) is slightly higher than with 4-MU-β-Xyl substrate (6.41 μM), presumably due to better protection against irreversible inhibition of GBA1 by the former substrate.

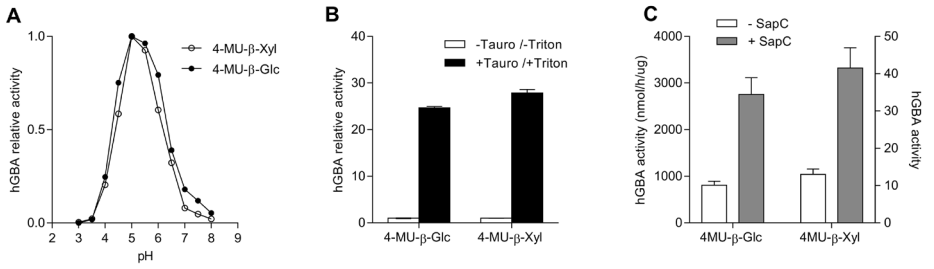

Figure 1. Cleavage of 4-MU-β-Glc and 4-MU-β-Xyl by recombinant hGBA1.

A. pH optimum of 4-MU release by GBA1 from 4-MU-β-Glc (closed circles) and 4-MU-β-Xyl (open circles). B. Stimulation by 0.2 % (w/v) taurocholate of 4-MU release from the substrates 4-MU-β-Glc (left) and 4-MU-β-Xyl (right). Expressed as 100 % is the activity measured in the absence of taurocholate at pH 5.2 with 0.1% (v/v) Triton X-100. C. Stimulation of 4-MU release from the substrates 4-MU-β-Glc (left axis) and 4-MU-β-Xyl (right axis) by recombinant saposin C at pH 4.5 in the presence of phosphatidylserine.

Table 1. Kinetic parameters hGBA1

hGBA1	**4-MU-β-Glc**	**4-MU-β-Xyl**
IC_{50} cyclophellitol (μM)	0.085 ± 0.002	0.061 ± 0.002
IC_{50} D-xylo-cyclophellitol (μM)	10.16 ± 1.03	6.41 ± 0.47
Km (mM)	0.76 ± 0.06	5.24 ± 1.04
Vmax (nmol/h/mg)	$1.23 \times 10^6 \pm 3.32 \times 10^4$	$1.88 \times 10^5 \pm 2.88 \times 10^4$
kcat/Km (mM/s)	25.03	0.55

Transxylosidase activity of GBA1.

We investigated recombinant GBA1 with respect to transxylosidase activity. For this, the enzyme was incubated for 16 hours with 4-MU-β-Glc or 4-MU-β-Xyl as donors and fluorescent 25-NBD-cholesterol as acceptor and next the products were analyzed by HPTLC and fluorescence scanning. Formation of fluorescent sterol metabolites occurred with both donors (Figure 2a). With 4-MU-β-Glc, glucosylated 25-NBD-cholesterol is formed as earlier described [10]. With 4-MU-β-Xyl, two novel fluorescent metabolites were detected, presumed to be mono- and di-xylosylated 25-NBD-cholesterol (Figure 2A).

Next, we used natural cholesterol as acceptor in the same assay with 4-MU-β-Xyl as donor and the formed products were analyzed by LC-MS/MS. In this way, formation of XylChol, Xyl_2Chol, and traces of Xyl_3Chol was detected (Figure 2B). In sharp contrast, incubation of GBA1 and cholesterol with 4-MU-β-Glc only renders GlcChol as product (Figure 2C).

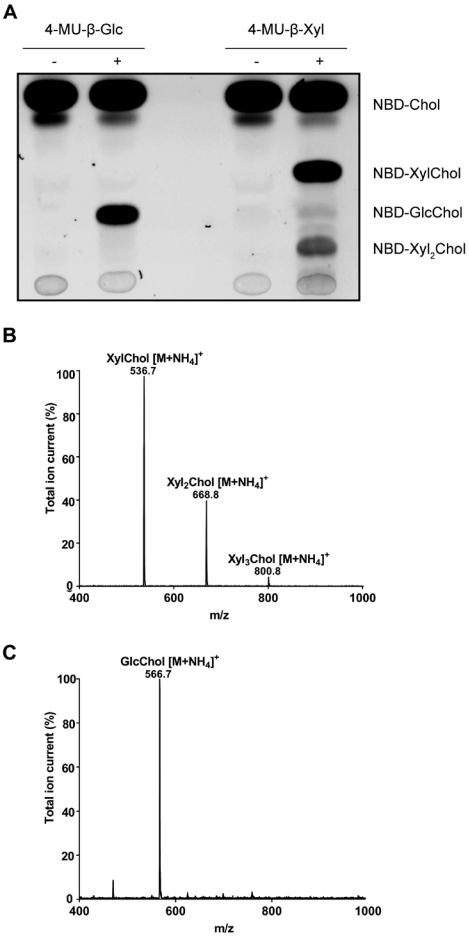

Figure 2. Transxylosylation and transglucosylation of cholesterol by GBA1.

A. HPTLC analysis of fluorescent products formed from 25-NBD-cholesterol following incubation with GBA1 in the presence of 4-MU-β-Glc or 4-MU-β-Xyl for 16h. B. LC-MS/MS analysis of products formed during 1h incubation of GBA1, cholesterol and 4-MU-β-Xyl. C. LC-MS/MS analysis of products formed during 1h incubation of GBA1, cholesterol and 4-MU-β-Glc.

Time dependence of glycosidase and transglycosidase activities of GBA1.

GBA1 and cholesterol were incubated with 4-MU-β-Glc or 4-MU-β-Xyl at 37 ˚C for different time periods. The release of 4-MU and formation of glycosylated products was determined. The formed GlcChol was already maximal after an incubation of 30 min and subsequently declined with time (Figure 3A). This suggests that the formed GlcChol is subject to subsequent hydrolysis by GBA1. In sharp contrast, XylChol showed no prominent reduction over time, and Xyl_2Chol was formed after a lag period (Figure 3B). This suggests that XylChol is hardly hydrolyzed and acts as acceptor for further xylosylation. This process continues with Xyl_2Chol acting as acceptor rendering Xyl_3Chol (Figure 3). A comparison of the release of 4-MU with concomitant formation of glycosylated sterol indicates that GBA1 shows considerably higher net transxylosylation than transglucosylation efficiency (Figure 3).

We next studied the outcome of the incubation of GBA1 and cholesterol with 4-MU-β-Xyl (3h) followed by 4-MU-β-Glc (1h). Formation of GlcXylChol (with m/z 698.5 > 369.3) was demonstrable at these conditions, again pointing to XylChol acting as excellent acceptor in glycosylation reaction (Supplemental Table 2).

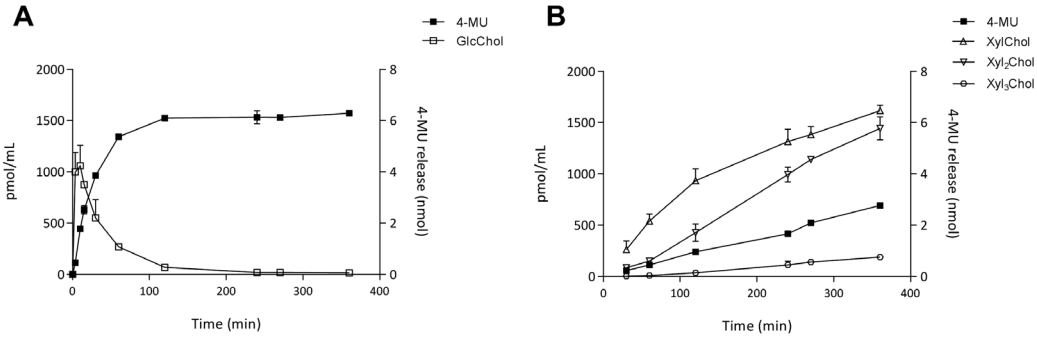

Figure 3. Glycosylation of cholesterol in time.

A. Release of 4-MU from 4-MU-β-Glc and concomitant formation of glucosylated cholesterol. B. Release of 4-MU from 4-MU-β-Xyl and concomitant formation of xylosylated cholesterol. GBA1 was incubated at pH 5.2 in the presence of taurocholate and Triton X-100 with 3.0 mM 4-MU-substrates for indicated times whereafter released 4-MU was determined and formed glycosylated cholesterol.

In vivo formation of xylosylated cholesterol.

To substantiate our *in vitro* findings, potential transxylosylation by cultured RAW264.7 cells exposed to 3.7 mM 4-MU-β-Xyl was investigated. Cells were incubated without or with the irreversible GBA1 inhibitor CBE either in the absence (Figure 4A) or presence of 10 μM U18666A (Figure 4B) to induce lysosomal accumulation of cholesterol [10]. Formation of XylChol, Xyl_2Chol and Xyl_3Chol was detected by LC-MS/MS. The levels of xylosylated cholesterols were markedly increased by the exposure of cells to U18666A and prohibited by prior inhibition of GBA1 with CBE. We next tested the possible involvement of UDP-glucose dependent GCS [27, 28] in formation of xylosylated cholesterols. HEK293 cells made deficient in GCS by CRISPR/Cas9 produced XylChol on a par to corresponding cells when exposed to 4-MU-β-Xyl and U18666A (Figure 4C and D).

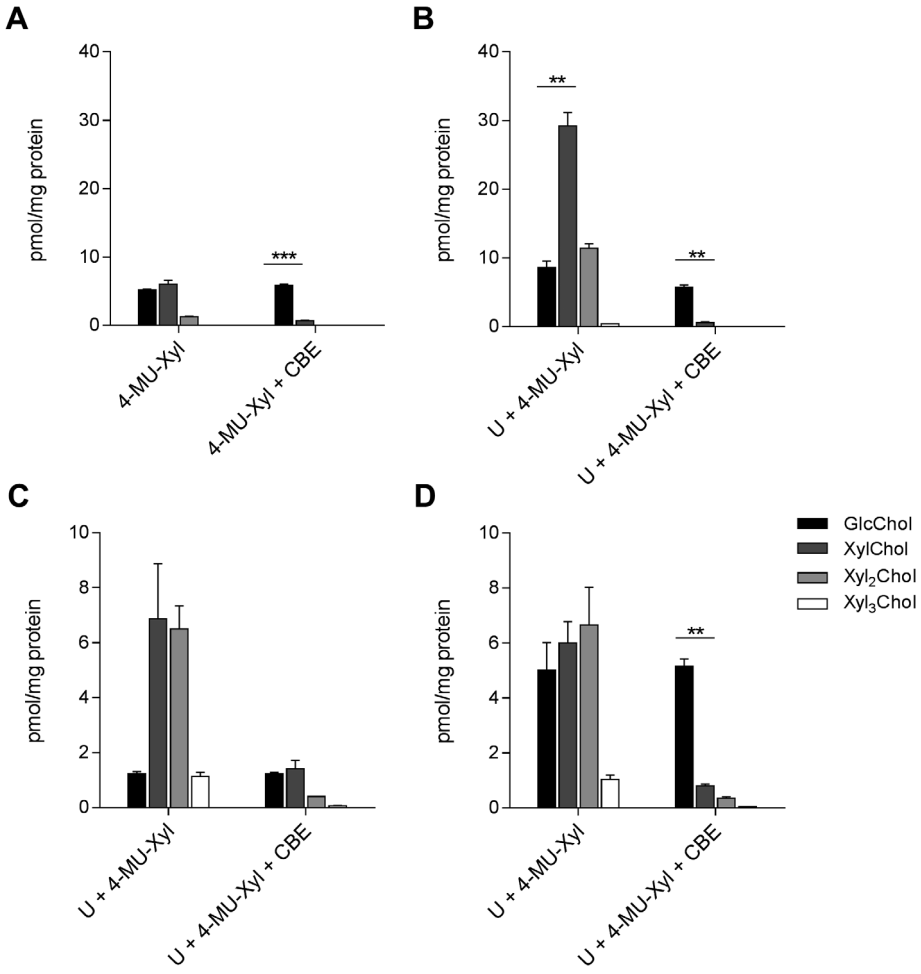

Figure 4. In vivo formation of xylosylated cholesterols.

A. LC-MS/MS detection of formed xylosylated and glucosylated cholesterols in RAW264.7 cells incubated with 3.7 mM 4-MU-β-Xyl for 24 hours in the presence/absence of CBE. B. Idem in the presence of 10 μM U18666A. C. LC-MS/MS detection of formed xylosylated and glucosylated cholesterols in HEK293 exposed to 3.7 mM 4-MU-β-Xyl and U18666A for 24 hours in the presence/absence of CBE. D. LC-MS/MS detection of formed glycosylated cholesterols in HEK293 cells deficient in GCS as in panel C. Significance of differences in formation of GlcChol and XylChol was determined and is indicated in the panels A-D.

Specificity of transxylosylation.

Finally, we studied potential transxylosylation by the two other human retaining β-glucosidases, GBA2 and GBA3. We earlier noticed that GBA2, but not GBA3, can mediate transfer of the glucosyl moiety from 4-MU-β-D-glucose to cholesterol or ceramide [10]. While this finding was recapitulated (Figure 5), concomitantly no xylosylation by GBA2 was detectable consistent with its inability to hydrolyze 4-MU-β-D-Xyl [6]. However, GBA3, albeit less prominent than GBA1, is able to hydrolyze 4-MU-β-Xyl as well as to transxylosylate cholesterol (Figure 5).

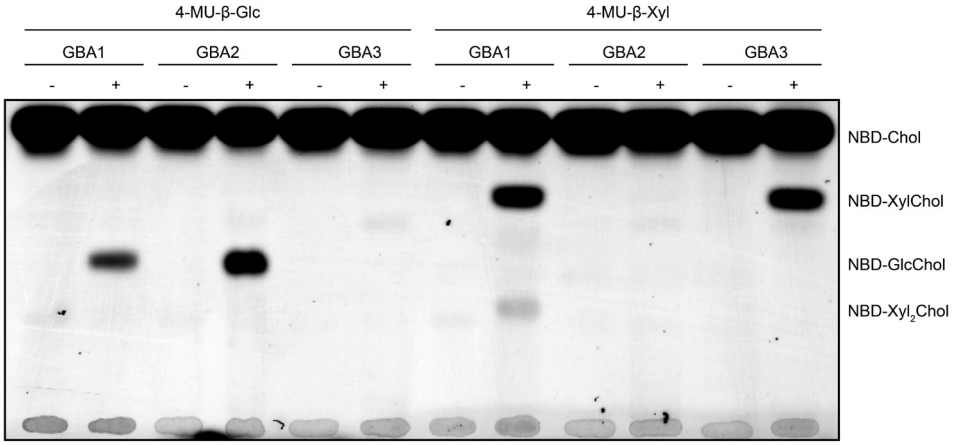

Figure 5. Lack of transxylosylation by GBA2.

HPTLC analysis of formation of glycosylated 25-NBD-cholesterol by β-glucosidases with 4-MU-β-Xyl and 4-MU-β-Glc as donor. Enzymes: recombinant hGBA1; lysate of HEK293 cells overexpressing GBA2; lysate of HEK293 cells overexpressing GBA3. Incubation for 16 hours with (+) or without (-) enzyme preparation.

Discussion

Our present investigation reveals an intriguing novel catalytic feature of human GBA1, the lysosomal glucocerebrosidase. First, we noted that GBA1 cleaves *in vitro* besides 4-MU-β-Glc also 4-MU-β-Xyl. Moreover, the enzyme uses both substrates as sugar donors in transglycosylation of cholesterol molecules. Next, we observed the generation of xylosylated cholesterol in living cells exposed to 4-MU-β-Xyl. Induction of lysosomal cholesterol accumulation in cells with U18666A increases formation of xylosylated cholesterols, a reaction prohibited by inactivation of GBA1 with the irreversible inhibitor conduritol B-epoxide. Remarkably, both *in vitro* and *in vivo*, GBA1 may even produce di-xylosyl-cholesterol using 4-MU-β-Xyl as sugar donor, a repetitive transglucosylation not seen with 4-MU-β-Glc as sugar donor [10]. The affinity of GBA1 for 4-MU-β-Glc as substrate for cleavage is higher than that for 4-MU-β-Xyl. Likewise, XylChol is a much poorer substrate for hydrolysis by GBA1 than GlcChol. Following exposure of GBA1 to cholesterol and 4-MU-β-Xyl, the concentration of XylChol steadily builds up and it starts to act as acceptor in a second round of transxylosylation, rendering Xyl_2Chol. Incubation of GBA1 and cholesterol with a mixture of 4-MU-β-Xyl and 4-MU-β-Glc leads to formation of GlcXylChol, further highlighting the suitability of XylChol as acceptor in transglycosylation by GBA1.

Net formation of XylChol by GBA1 exceeds that of GlcChol, a phenomenon that can be ascribed to the lower rate of hydrolysis of XylChol than GlcChol. At present, we can't exclude the possibility that a xylose covalently bound to the catalytic nucleophile E340 of GBA1 is also somehow transferred more efficiently to cholesterol than a covalently bound glucose. Further insight in this might be obtained by crystallography employing β-glucose and β-xylose configured cyclophellitol-epoxides covalently bound to E340-GBA1. Of note, Aerts and co-workers earlier noted also relative higher transxylosidase than transglucosidase efficiency of a β-D-glucosidase from *Stachybotrys atra* [27], quite comparable to our findings with GBA1.

The physiological relevance of transxylosylation by GBA1 is presently entirely unclear. Mass spectrometry suggests the presence of XylChol (m/z 536.5 > 369.3) in liver of *Npc1-/-* mice (data not shown). Of note, in the same livers accumulation of GlcChol has prior been detected [10]. Warranted is definitive confirmation of XylChol in NPC liver by NMR analysis of purified lipid. In this connection, a key question is whether physiological xyloside donors occur. Several β-xylosidic compounds are known to be produced by plants and their uptake via food is a priori not excluded [28]. β-D-xylosyl moieties are also present in endogenous proteoglycans from which xylosyl-peptides are formed during lysosomal degradation. It is unclear whether these are suitable donors for GBA1-mediated formation of xylosylated sterols. It can presently not be excluded that another UDP-xylose dependent xylosyltransferase may generate hitherto unknown β-xyloside donors to be used in transxylosylation. One such candidate is UGT3A2, a UDP-xylose-utilizing glycosyltransferase shown to glycosylate a variety of hydrophobic structures *in vitro* [29]. Moreover it should be investigated whether other lipids besides cholesterol may act as acceptors in transxylosylation by GBA1 [10].

Our investigation further has revealed that GBA3, a cytosolic glucosidase implicated in metabolism of xenophobic glycosides [17], also shows xylosidase and transxylosidase activity. In contrast, the β-glucosidase GBA2 shown to be a potent transglucosidase has no significant activity towards β-xyloside substrates. Apparently the pendant CH_2OH group in glucoside substrates contributes crucially to the interaction of substrate with GBA2. The importance of the presence of the additional CH_2OH group in glucose is also suggested by the much lower affinity of GBA2 for the inhibitor conduritol-B-epoxide as compared to cyclophellitol (with the pendant CH_2OH group).

In conclusion, human GBA1 is more versatile in catalysis as hitherto considered. Investigation

of the (patho)physiological relevance of various reactions catalyzed by GBA1 is needed to further complete understanding of the full symptomatology of Gaucher disease [7, 8] and other conditions for which abnormal GBA1 imposes a risk such as multiple myeloma and α-synucleinopathies like Parkinsonism and Lewy-body dementia [30, 31].

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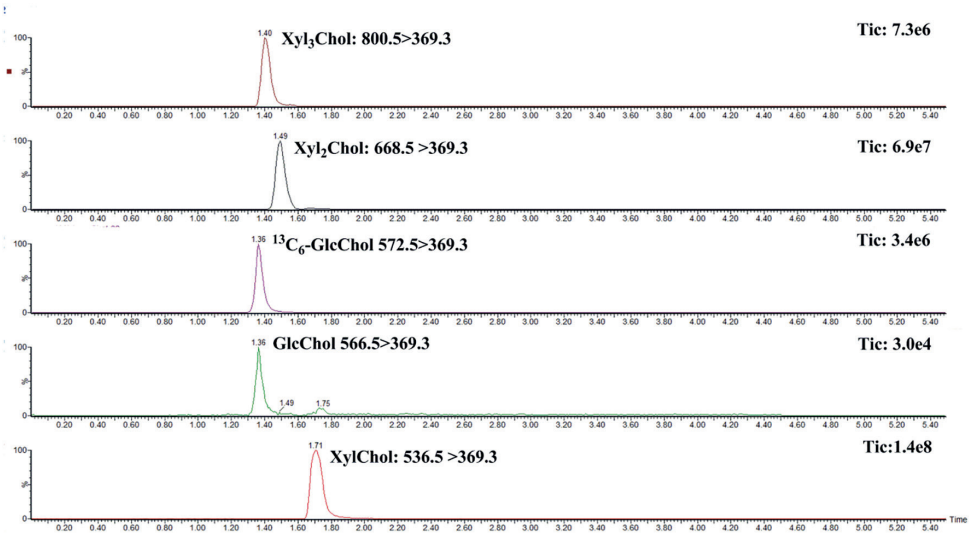
Supplemental data



Supplemental Figure 1. *Chromatogram of XylChol, Xyl_2Chol, Xyl_3Chol, GlcChol and $^{13}C_6$-GlcChol.*

Supplemental Table 1. *MS/MS instrument parameters.*

Capillary voltage	3.50 KV
Cone voltage	20 V
Source temperature	150 °C
Desolvation temperature	450 °C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision voltage	15 V
Type	Multiple reaction monitoring
Ion mode	ES^+ (electrospray positive)
Dwell time	0.1 s
Interchannel delay	0.005 s
Interscan delay	0.005 s
Transitions:	**RT(min.):**
GlcChol	1.36
$^{13}C_6$-GlcChol	1.36
XylChol	1.71
Xyl_2Chol	1.49
Xyl_3Chol	1.40
Fit weight	None
Smooth method	Mean
Smooth width	2

Supplemental Table 2. *Formation of hybrid GlcXylChol following incubation of GBA1 with 1:1 mixture of 4-MU-β-Xyl and 4-MU-β-Glc.*

MRM transitions	**4-MU-Xyl (3h)** **4-MU-Glc (1h)**	**pmol/mL**
536.5 > 369.3	XylChol	631.56
566.5> 369.3	GlcChol	293.20
668.5> 369.3	Xyl_2Chol	76.34
698.5> 369.3	XylGlcChol	10.58
800.5> 369.3	Xyl_3Chol	3.34
830.5> 369.3	Xyl_2GlcChol	0.42