Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

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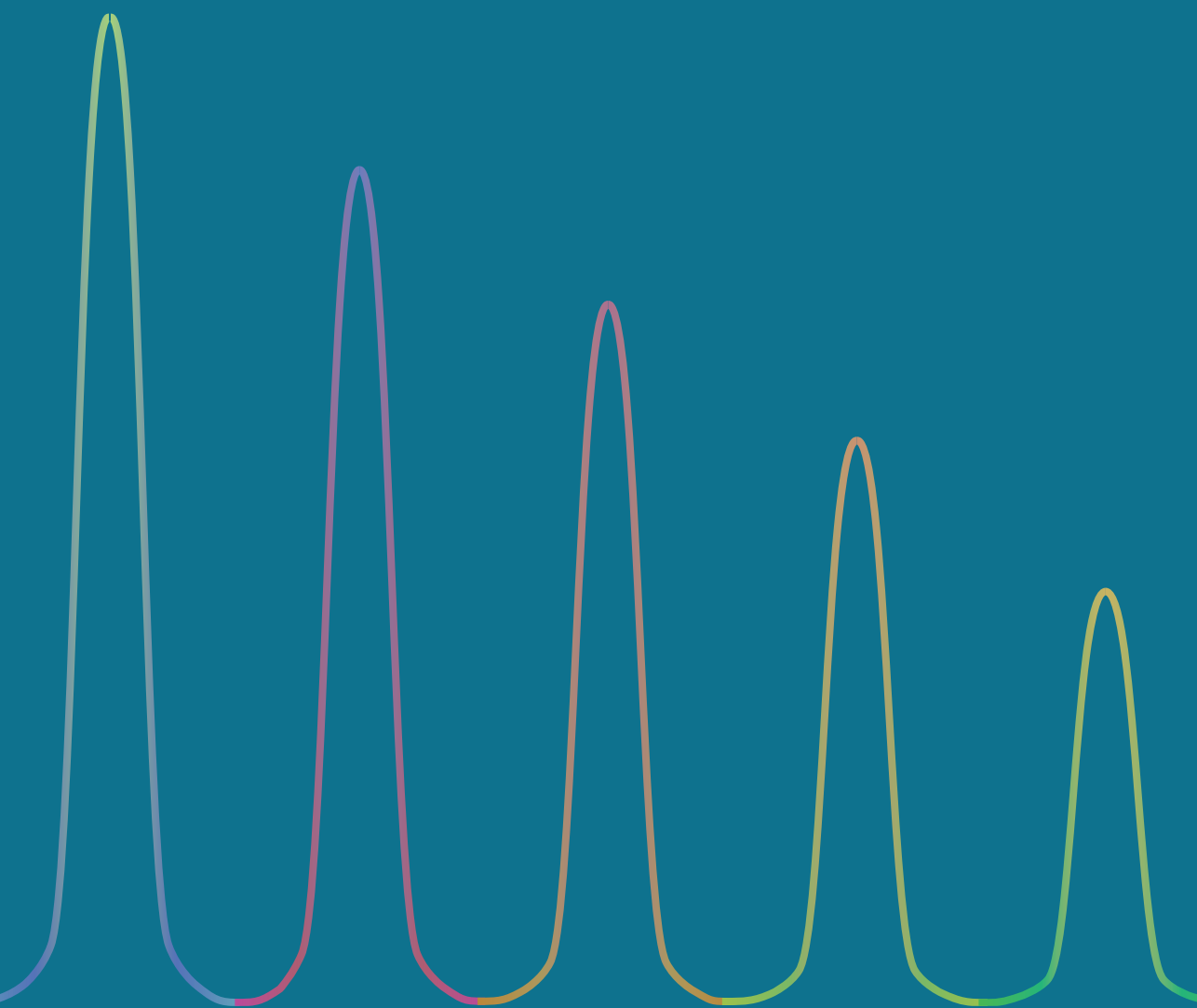

Chapter 5

Potential role of GBA3 in formation and degradation of glycosylated metabolites

To be incorporated in manuscript in preparation

Chapter 5 - Potential role of GBA3 in formation and degradation of glycosylated metabolites

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Contributions:
H.N.J.M. : author, performed described experimental biochemistry and analytical chemistry
P.W. : (BioSyn, LIC); synthesis of (isotope labeled) GlcChol, Glc7DHC and $GlcD_3$
K.K. : synthesis of (isotope labeled) GlcChol and GlcDesm
M.M. : advise with LC-MS/MS
M.J.F. : advise with sample preparation
M.A. : co-supervision
J.M.A. : supervision

Abstract

The recently recognized transglucosylation ability of by the cellular β-glucosidases, lysosomal glucocerebrosidase (GBA) and membrane-associated nonlysosomal glucosylceramidase GBA2, have prompted studies on this for the broad-specific β-glucosidase (GBA3). We studied the capacity of GBA3 *in vitro* to hydrolyze β-glucosides as well as its ability to transglucosylate acceptors. We investigated, with our sensitive quantitative LC-MS/MS method, the *in vitro* formation of GlcChol by GBA3 and the degradation of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. During our study we observed that GBA3 was not able to form GlcChol, nor that GBA3 was able to degrade any of the studied metabolites. The negative findings warrant use of larger amounts of a purified recombinant GBA3.
It is however conceivable that GBA3 is indeed unable to form and degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. The obtained results stimulate further research on GBA3 to synthesize and degrade galactosylated or xylosylated cholesterol, 7-dehydrocholesterol, D_3 (cholecalciferol) or desmosterol.

Introduction

The broad-specific β-glucosidase (GBA3), also known as klotho-related protein (KLrP), is known to be cytosolic and present in kidney, liver, spleen, intestine and lymphocytes of mammals. It is thought to be involved in hydrolysis of xenobiotic glycosides [1, 2]. The GH1 enzyme is thought to be most likely involved in detoxification of glucosylated xenobiotics. Via drinking water, food, medical treatment or pollution these substances are absorbed into the body and require detoxication [3]. Plants are known to contain various α- and β-glucosidases, galactosidases and xylosidases [4]. As we eat plants, these glucosylated metabolites enter our body as glucosylated xenobiotics. GBA3, as broad-specific glycosidase, hydrolyzes substrates with a variety of an α-arabinose, β-glucose, β-galactose or β-xylose moiety linked to a hydrophobic group [5]. Artificial substrates such as 4-methylumbelliferyl-β-D-glucoside and C_6-NBD-glucosylceramide are hydrolyzed by GBA3, but naturally occurring lipids like glucosylceramide (GlcCer) and glucosylspingosine seem poor substrates [1, 2]. GBA3 has been studied in relation to Gaucher disease (GD). The underlying cause of GD is deficiency of glucocerebrosidase (GBA), the enzyme responsible for the hydrolysis of GlcCer into glucose and ceramide. Deficiency of GBA causes lysosomal storage of GlcCer in tissue macrophages, resulting in a broad spectrum of clinical presentations [6-10]. GBA3 is thought not to have any involvement in the clinical manifestation of non-neurological (type 1) GD [1].

The 469 amino acid GBA3 is encoded by the GBA3 gene on the 4p15,31 locus [11-13]. The crystal structure reveals the presence of a characteristic TIM barrel of retaining glycosidase, potentially capable of transglycosylation [14, 15]. GBA3 uses a similar double displacement mechanism as the enzymes GBA and GBA2 capable of transglucosylation [2, 16]. GBA3 has two catalytic glutamate residues in its active site (Glu 165 and Glu 373) [2]. The protonated aglycone departs after nucleophilic attack of the glutamate residue leading to an α-linked covalent glycosyl-enzyme intermediate. The crystal structure of the covalent glycosyl intermediate was elucidated and the double displacement hydrolysis mechanism of GBA3 was confirmed [15].

We examined the ability of GBA3 to perform transglucosylation as well as its ability to glucosylated metabolites that were earlier detected in human tissue.

Results

Formation and degradation of glucosylated metabolites by GBA3

Previous investigations did not point to transglycosylation activity of GBA3 [17]. This was confirmed by the analysis of GlcChol formation via transglycosylation by GBA3 (Figure 1). For this, homogenates of cells overexpressing GBA3 were incubated with cholesterol (Chol) as acceptor and either 4MUGlc or GlcCer as sugar donor. The activity of GBA and GBA2 in the homogenates was included or excluded by adding the following inhibitors: final concentration of 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or *N*-adamantine-methyloxypentyl-deoxynojirimycin 1 µM AMP-DNM (preferential GBA2 inhibitor). The sugar donor, either GlcCer (0.1 mM) or 4MUGlc (3.7 mM), was combined with 0.1 mM of Chol. The reaction was incubated for 1 hour at 37°C in 100 mM HEPES buffer (pH 7.0). The formation of GlcChol was monitored by LC-MS/MS.

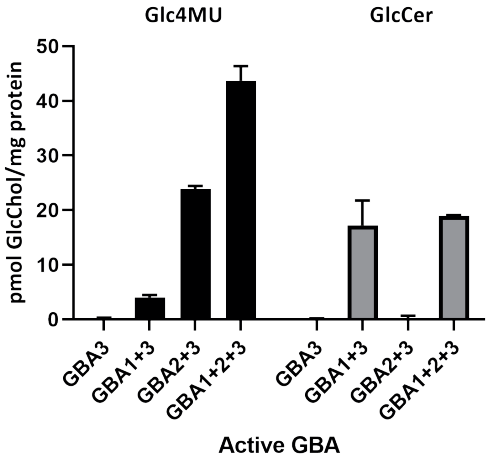

Figure 1. In vitro formation of GlcChol. *Lysates of cells overexpressing GBA3 were incubated for varied times with cholesterol (Chol) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol was measured by LC-MS/MS (pmol/mg protein). GBA and GBA2 was included or excluded by making use of inhibitors, 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or 1 µM AMP-dNM (GBA2 specific inhibitor).*

We next investigated the ability of GBA3 to degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. These metabolites were synthesized at the Department Bi-organic Synthesis. Each of the compounds was incubated at 4 µM at 37°C for varied times with homogenates of cells overexpressing GBA3. To exclude action of GBA and GBA2 the homogenates were pretreated with the irreversible GBA inhibitor ME656 or the preferential GBA2 inhibitor AMP-DNM. The reduction of the metabolites during a 4 hour incubation was monitored by LC-MS/MS. No degradation of the glucosylated metabolites observed (Figure 2).

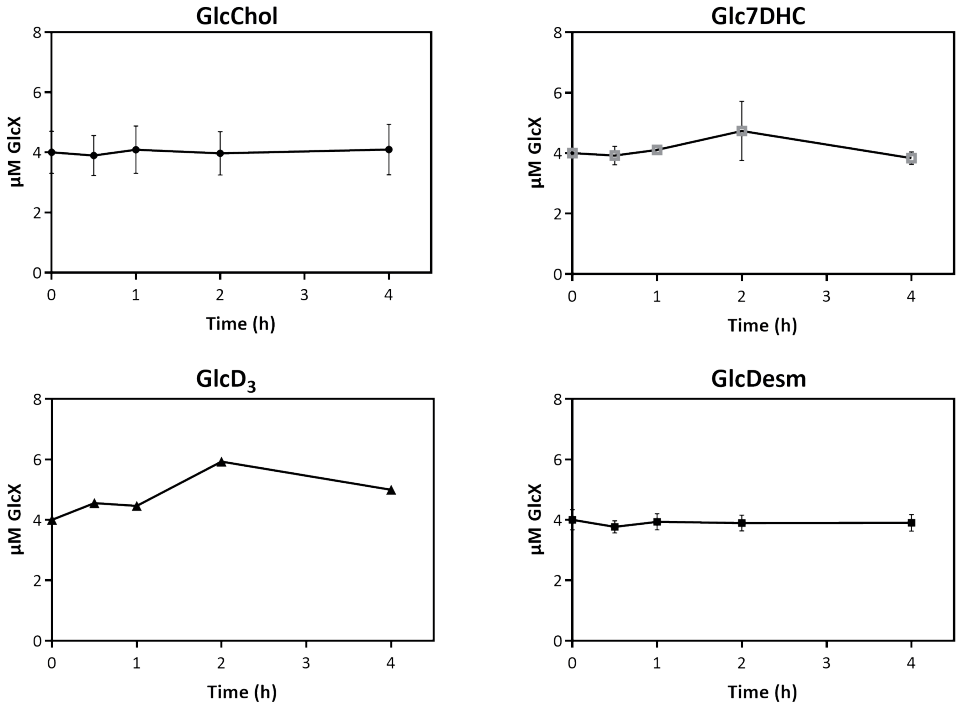

Figure 2. Degradation by GBA3. *GBA3 was incubated for varied times with 4 µM GlcChol, 4 µM Glc7DHC, 4 µM $GlcD_3$ or 4 µM GlcDesm. Degradation of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm was measured by LC-MS/MS (µM) and corrected for amount of protein. GBA and GBA2 activity was excluded by using the inhibitors: 50 nM ME656 (GBA1 specific inhibitor) and/or 1 µM AMP-dNM (preferential GBA2 inhibitor).*

Discussion

The obtained data indicate that GBA3 is not able to perform transglucosylation of cholesterol to generate GlcChol. Moreover, GBA3 seems not able to significantly degrade the glucosylated metabolites GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. Previously presented data shows that the latter compounds are all endogenous. Lack of metabolism of GBA3 might be contributing to this, as GBA3 is known to be active towards glycosylated metabolites with a rigid, planar, aglycon, such as quercetin [15, 18]. The aglycon specificity of hCBG has mainly been studied for xenobiotic β-D-glycosides [19]. β-D-glucosides of isoflavones, flavonols and flavones were identified as good substrates. A flat aromatic molecule is a favorable substrate compared to aliphatic analogues. Glucosides of dihydrochalcones (phlorizin) and anthocyanins were not substrates [18]. GBA3 hydrolyzes cyanogenic glucosides and phenolic glucosides [19]. Modelling and crystallography with recombinant GBA3 might confirm the observed lack of metabolism of GBA3 for GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. Quercetin-glucoside is in this case good positive control.

In addition, investigations of the activity of GBA3 towards galactosylated or xylosylated forms of metabolites is of interest. In plants xylose is a major sugar and several plant β-xylosides compounds are known [20]. Via food uptake, some of these might enter the body and might act as xylose-donors for transxylosylation reactions [21, 22].

Most importantly, larger scale production and purification of GBA3 seems warranted to definitively exclude (or establish) a role of the enzyme in the metabolism of glycosylated metabolites in humans. Such role would be relevant in view of the frequent inherited defect in GBA3 [1].

Acknowledgements

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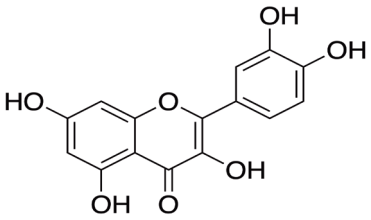
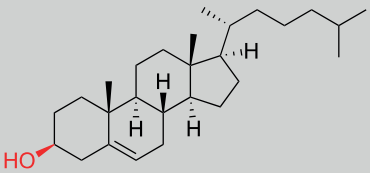
Table 1. Chemical structures of rigid planar hydrophobic compounds. *Presented are compounds Quercetin, of which the glucosylated compounds fits in the pocket of GBA3 [15], cholesterol (chol), 7-dehydrocholesterol (7DHC), Desmosterol (Desm), vitamin D (cholecalciferol, D_3) of which the glycosylated compounds do most probably not fit the pocket of GBA3.*

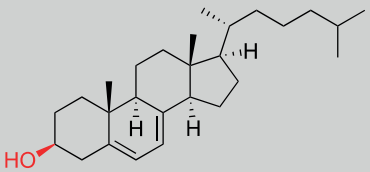
Quercetin



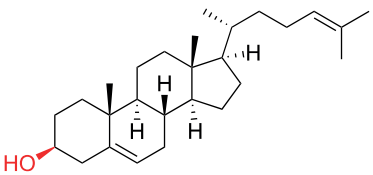
Chol



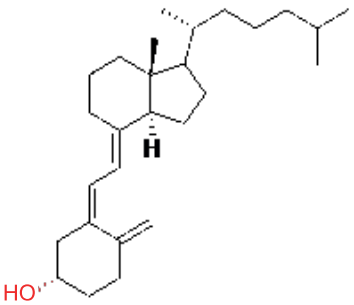
7DHC



Desm



D_3



Experimental procedures

Materials

As in previous work the following pure grade chemicals were used: cholesterol (Sigma-Aldrich, St Louis, MO, USA), D-glucosyl-β-1,1'*N*-oleoyl-D-*erythro*-sphingosine (C18:1-GlcCer) (Avanti Polar Lipids, Alabaster, AL, USA), 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) (Glycosynth™, Winwick Quay Warrington, Cheshire, England). The department of Bio-organic Synthesis (University of Leiden, The Netherlands) synthesized the used inhibitors following previously described procedures: GBA1 inhibitor ME656 [23], Conduritol B Epoxide (CBE, GBA1 specific inhibitor) [24] and AMP-DNM (GBA2 specific inhibitor) [25]. As specific lipid substrates were used: $^{13}C_6$-β-GlcChol [24], GlcChol, $^{13}C_6$-β-Glc7DHC, Glc7DHC and $GlcD_3$, $^{13}C_6$-β-GlcDesm and GlcDesm synthesized at the Bio-organic Synthesis department (University of Leiden, The Netherlands).

As source of GBA3, lysates of HEK293T-cells overexpressing the enzyme were used.

For LC-MS/MS the following pure grade solvents were used: ethanol (Honeywell Riedel-de Haën™, Muskegon, USA), LC-MS-grade methanol, 2-propanol, water and HPLC-grade chloroform (Biosolve), LC-MS butanol (Merck KGaA, Darmstadt, Germany) and LC-MS quality ammonium formate (Sigma-Aldrich, St Louis, MO, USA).

Methods

In vitro assay of transglucosylation activity. Lysates of HEK293T cells overexpressing GBA3 were used to determine transglucosylation activity of the enzyme GBA3. Specific inhibitors were used to inhibit the enzymatic activity of GBA and GBA2 in the lysates. The assays were performed as described earlier [17] with the following modifications. The samples contained 40 µL homogenate of GBA3 overexpressing cells, to which was added a final concentration of 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or 1 µM AMP-DNM (GBA2 specific inhibitor) or 100 mM HEPES buffer (pH 7.0). The glucose donor was either 4-methylumbelliferyl-β-D-glucoside (4MUGlc) or glucosylceramide (GlcCer). The final concentrations in the reaction were 100 µM C18:1-GlcCer or 2.8 mM 4MUGlc. As final concentration of acceptor 100 µM of Chol was added to the reaction.

The transglucosylation reaction was carried out in the presence of additional 200 µL 100 mM HEPES buffer (pH 7.0) and 12.5 µL of ethanol. After 1h incubation at 37°C, the transglucoylsation reaction was terminated by putting samples on ice, the samples were subjected to lipid extraction according to Bligh and Dyer [26] by addition of methanol, chloroform and water (final volumes: 1:1:0.9, v/v/v). As internal standard 50 µL aliquots of 0.1 pmol/µL $^{13}C_6$-β-GlcChol each in methanol were added. The lower lipid phase was taken to dryness under vacuum at 45°C in a *speed FAC concentrator plus*. The isolated lipids were purified by butanol/water

extraction (1:1, v/v) and the upper phase was taken to dryness under vacuum at 45 °C in a *speed FAC concentrator plus*. The isolated lipids were resolved in methanol and analysed by LC-MS/MS.

Degradation of GlcChol, Glc7DHC and $GlcD_3$ and GlcDesm. The ability of homogenates of HEK293T cells overexpressing GBA3 to degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm was monitored over time. Therefore 4 µM of GlcChol, Glc7DHC, $GlcD_3$ or GlcDesm was added to 40 µL of homogenate of GBA3 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) and 1 µL of 1 µM of AMP-DNM or 100 mM HEPES buffer (pH 7.0). The reaction was carried out in the presence of additional 5 µL of ethanol. All samples were prepared for LC-MS/MS as described previously and measured by LC-MS/MS for GlcChol, Glc7DHC, $GlcD_3$ or GlcDesm levels.

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

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