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Ambroxol-induced rescue of defective glucocerebrosidase is associated with increased LIMP-2 and saposin C levels in *GBA1* mutant Parkinson's disease cells

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ABSTRACT

Heterozygous mutations in *GBA1* gene, encoding for lysosomal enzyme glucocerebrosidase (GCase), are a major risk factor for sporadic Parkinson's disease (PD). Defective GCase has been reported in fibroblasts of *GBA1*-mutant PD patients and pharmacological chaperone ambroxol has been shown to correct such defect. To further explore this issue, we investigated GCase and elements supporting GCase function and trafficking in fibroblasts from sporadic PD patients — with or without heterozygous *GBA1* mutations — and healthy subjects, in basal conditions and following in vitro exposure to ambroxol. We assessed protein levels of GCase, lysosomal integral membrane protein-2 (LIMP-2), which mediates GCase trafficking to lysosomes, GCase endogenous activator saposin (Sap) C and parkin, which is involved in degradation of defective GCase. We also measured activities of GCase and cathepsin D, which cleaves Sap C from precursor prosaposin. GCase activity was reduced in fibroblasts from *GBA1*-mutant patients and ambroxol corrected this defect. Ambroxol increased cathepsin D activity, GCase and Sap C protein levels in all groups, while LIMP-2 levels were increased only in *GBA1*-mutant PD fibroblasts. Parkin levels were slightly increased only in the PD group without *GBA1* mutations and were not significantly modified by ambroxol. Our study confirms that GCase activity is deficient in fibroblasts of *GBA1*-mutant PD patients and that ambroxol corrects this defect. The drug increased Sap C and LIMP-2 protein levels, without interfering with parkin. These results confirm that chemical chaperone ambroxol modulates lysosomal markers, further highlighting targets that may be exploited for innovative PD therapeutic strategies.

Introduction

Heterozygous mutations in the *GBA1* gene have been recently identified as a major genetic risk factor for the development of sporadic Parkinson's disease (PD) (Sidransky et al., 2009; Sidransky and Lopez, 2012; Asselta et al., 2014) and dementia with Lewy bodies (Nalls et al., 2013; Asselta et al., 2014). Homozygous mutations in the *GBA1* gene cause Gaucher disease (GD); heterozygous mutations occur in 5–10% of sporadic PD cases (Schapira and Gegg, 2013) and impair folding and activity of glucocerebrosidase (GCase), the lysosomal enzyme encoded by the *GBA1* gene. These alterations may impact on lysosomal function and, consequently, on cellular clearance pathways, such as autophagy. Since alpha-synuclein is a substrate of chaperone-mediated autophagy, any alteration in this proteolytic mechanism may favor the pathological intracellular accumulation of this protein and formation of Lewy bodies in PD brains (Alvarez-Erviti et al., 2010; Xilouri and Stefanis, 2015). Indeed, growing evidence is supporting a role for GCase dysfunction in the process of alpha-synuclein accumulation, thereby strengthening the link with PD pathogenesis (Bae et al., 2015; Siebert et al., 2014; Sardi et al., 2015; Schapira, 2015).

Reduced GCase activity, correlated with disease severity, has been detected in fibroblasts derived from patients with GD (Bendikov-Bar et al., 2013). More recently, McNeill et al. (2014) reported reduced activity and protein levels of GCase in fibroblasts from *GBA1* heterozygous mutation carriers, with and without PD. In both GD and *GBA1*-mutant PD patients, these defects were corrected by exposing fibroblasts to ambroxol hydrochloride, a small molecule known for its expectorant and anti-inflammatory activity that may function as a molecular chaperone (Maegawa et al., 2009; McNeill et al., 2014). Ambroxol improved lysosomal biochemistry by modulating the expression of transcription factor EB (TFEB)-associated genes and endoplasmic reticulum (ER) markers (McNeill et al., 2014). Based on these results, ambroxol has been proposed as a potential disease-modifying treatment for PD. In this perspective, the mechanism of action of ambroxol should be further

Abbreviations: PD, Parkinson's disease; GD, Gaucher disease; GCase, glucocerebrosidase; Sap C, saposin C; PSAP, prosaposin; LIMP-2, lysosomal integral membrane 2; ER, endoplasmic reticulum; TFEB, transcription factor EB; GSK3, glycogen synthase kinase 3; UPS, ubiquitin proteasome system.

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characterized. For example, the impact of ambroxol on cellular proteostatic mechanisms and how this may reverberate on GCase efficiency should be clarified.

GCase function and targeting to the lysosomes are supported by endogenous transporters and co-factors. Lysosomal integral membrane protein-2 (LIMP-2), a type III glycoprotein encoded by *SCARB2* gene, is the receptor involved in the lysosomal transport of GCase (Reczek et al., 2007; Rothaug et al., 2014). Saposin C (Sap C) is a substrate presenting co-factor essential in the GCase-dependent hydrolysis of glucosylceramide, which also protects GCase from proteolytic breakdown (Siebert et al., 2014). Sap C — which is defective in a rare variant of GD (Tatti et al., 2011; Motta et al., 2014) — belongs to a family of four small glycoproteins (saposins A–C) originating from the sequential lysosomal proteolysis of precursor prosaposin (PSAP) (Misasi et al., 2009; Hiraiwa et al., 1997; Tamargo et al., 2012). The cleaving enzyme is cathepsin D, a lysosomal aspartyl protease also involved in the processing of alpha-synuclein (McGlinchey and Lee, 2013; Crabtree et al., 2014). Interestingly, mutations in LIMP-2, as well as Sap C deficiency, have been investigated as genetic modifiers in GD and, more recently, in synucleinopathies (Siebert et al., 2014; Sardi et al., 2015).

In fibroblasts from GD patients or PD patients carrying heterozygous *GBA1* mutations, misfolded GCase is retained in the ER, thereby causing ER stress (Westbroek et al., 2011; Tan et al., 2014). ER stress activates the unfolded protein response and modulates proteins that are typically at the cross-road of proteostasis, cell metabolism and viability, such as glycogen synthase kinase 3 (GSK3) and parkin. GSK3 — with the associated signaling pathway involving serine/threonine protein kinase AKT (also known as protein kinase B) and mTOR — is linked to ER stress, which suppresses GSK3-Akt signaling (Chen et al., 2011; Golpich et al., 2015). More importantly, recent reports show that GSK3 inhibition plays a pivotal role in the regulation of lysosomal biogenesis and function in different models of Alzheimer's disease and tauopathies (Nijholt et al., 2013; Parr et al., 2012). Moreover, Ballabio's group has shown that mTOR, one of GSK3 downstream target kinases, interacts with and phosphorylates TFEB on the lysosomal membrane, thereby preventing its translocation to the nucleus, further indicating a connection between these pathways (Settembre et al., 2012). In parallel, parkin, a PD-related E3 ubiquitin ligase whose expression is regulated also by ER stress (Bouman et al., 2011), is involved in mitophagy (Calì et al., 2013) and in the ubiquitination and proteasomal degradation of several substrates, including mutant GCase (Ron et al., 2010; Bendikov-Bar et al., 2014).

We have previously reported proteolytic defects in lymphocytes and fibroblasts from sporadic PD patients, which included reduced proteasome 20S activity and increased levels of parkin and poly-ubiquitinated proteins (Blandini et al., 2006; Ambrosi et al., 2014). The objective of this study was to analyze the impact of *GBA1* mutations in the context of PD by investigating GCase and specific lysosomal factors supporting GCase activity in fibroblasts of PD patients with or without *GBA1* heterozygous mutations or healthy controls. Moreover, we sought to obtain further information on the effects of ambroxol in this context, to confirm its potential as a compound targeting lysosomal dysfunctions that may be central to PD pathogenesis.

Our results confirm that GCase is deficient in *GBA1*-mutant PD cells and that ambroxol enhances GCase activity and protein levels; this effect is associated with increased levels of GCase transporter LIMP-2 and co-factor Sap C.

Subjects and methods

Patients and fibroblast cultures

Fibroblasts were generated from skin biopsies of the upper medial arm of ten sporadic PD patients and seven age- and gender-matched healthy controls (Table 1). Within the PD group, five patients carried L444P (two cases) or N370S (three cases) heterozygous mutations in the *GBA1* gene (PD GBA+); these fibroblasts were obtained from the Telethon Network of Genetic Biobanks (Telethon, Italy). In the other five patients (PD GBA−) and in controls, *GBA1* mutations or polymorphisms were excluded by sequencing all 11 exons of the *GBA1* gene with Genetic Analyzer 3130xl (Applied Biosystems) and comparing data with a reference sequence available at www.ncbi.com (RefSeqGene:NG_009783.1) using Sequencher 4.8 software (GeneCodes, Ann Arbor, MI, USA). The research protocol was approved by the Ethic Committee of the "C. Mondino" National Neurological Institute and informed consent was obtained from all subjects.

Table 1
Clinical and demographic data of subjects enrolled for the study.

	Controls	PD GBA−	PD GBA+
n (gender)	*n* = 7 (3 M, 4 F)	*n* = 5 (3 M, 2 F)	*n* = 5 (3 M, 2 F)
Age (years)	62.8 ± 4.8	65.6 ± 3.1	55.4 ± 11.4
Age at onset (years)	–	51 ± 5.6	42.2 ± 8
Disease duration (years)	–	17.3 ± 3.7	13.2 ± 4.7
UPDRS scale III	–	19.5 ± 3	9.8 ± 7

Two GBA+ PD patients carried the L444P mutation and three the N370S mutation in the *GBA1* gene. All patients were under treatment with levodopa, except one GBA+ patient who was not taking any dopamine-based medication. The five GBA− patients were also taking dopamine agonists as adjunctive therapy. Unified PD rating scale (UPDRS) scores (part III) are reported in the ON phase. Results are expressed as mean ± SD.

All fibroblast strains were cultured in RPMI 1640(Sigma) complemented with 1% streptomycin and penicillin antibiotics and 20% fetal bovine serum (FBS, Sigma). Cells used in the experiments were grown and expanded in flasks up to a maximum of thirteen passages. Cells were treated for 5 days with 60 μM ambroxol (Sigma), according to previous data from McNeill et al. (2014). Ambroxol was dissolved in DMSO and a frozen stock (20 mM) was prepared. For each experimental round, all cell lines were kept in culture, treated and processed together. The medium was changed on the third day during the 5-day session and ambroxol-containing medium was prepared fresh each time.

Western blot analysis

Protein lysates were obtained by resuspending fibroblast pellets in ice-cold lysis buffer (CelLytic, Sigma) containing diluted phosphatase (1:10, Roche) and protease inhibitors (1:25, Roche). After centrifugation, the supernatant was collected and protein concentration was measured using a Bicinchoninic Acid Protein Assay (Sigma). Protein lysates were run on 4–12% gels, transferred onto nitrocellulose membranes (Invitrogen) and western blot was performed. Membranes were blocked (Odyssey blocking buffer, LiCor) and incubated overnight with the following primary antibodies: actin (1:2000), ubiquitin (1:1000), AKT1 (1:500), Sap C (1:1000), GSK3α/β (1:500), GSK3α-Phospho(Ser21) (1:500) (Santa Cruz); GCase (1:1000), Sigma; mTOR (1:1000), phospho-mTOR (1:500), parkin (1:500), phospho-Akt(Ser473) (1:500) and GSK3β-Phospho(Ser9) (1:500) (Cell Signalling); LIMP-2 (1:1000) (Novus Biologicals). As secondary antibodies, IRDye® 700 goat anti-mouse, IRDye® 800 goat anti-rabbit (1:10000) (LiCor, Biosciences) were used. Image analysis of western blots was performed using the fluorescent near-infrared Odyssey® scanner and software (LiCor, Biosciences) and fluorescence was normalized with the corresponding actin signal.

Quantitative real-time PCR

Real-time PCR (rtPCR) was used to quantify mRNA for *GBA1* gene. The expression of *GBA1* transcript was normalized to that of housekeeping gene actin as in McNeill's (2014) work. Total RNA extraction was performed with RNeasy Plus mini kit (Qiagen). The RNA concentration was determined by NanoDrop spectrophotometer ND-1000 (Thermo Scientific) and cDNA was synthesized using the iScript™ cDNA