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Glucocerebrosidase inhibition causes mitochondrial dysfunction and free radical damage

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ABSTRACT

Mutations of the gene for glucocerebrosidase 1 (*GBA*) cause Gaucher disease (GD), an autosomal recessive lysosomal storage disorder. Individuals with homozygous or heterozygous (carrier) mutations of *GBA* have a significantly increased risk for the development of Parkinson's disease (PD), with clinical and pathological features that mirror the sporadic disease. The mechanisms whereby *GBA* mutations induce dopaminergic cell death and Lewy body formation are unknown. There is evidence of mitochondrial dysfunction and oxidative stress in PD and so we have investigated the impact of glucocerebrosidase (GCase) inhibition on these parameters to determine if there may be a relationship of *GBA* loss-of-function mutations to the known pathogenetic pathways in PD. We have used exposure to a specific inhibitor (conduritol-β-epoxide, CβE) of GCase activity in a human dopaminergic cell line to identify the biochemical abnormalities that follow GCase inhibition. We show that GCase inhibition leads to decreased ADP phosphorylation, reduced mitochondrial membrane potential and increased free radical formation and damage, together with accumulation of alpha-synuclein. Taken together, inhibition of GCase by CβE induces abnormalities in mitochondrial function and oxidative stress in our cell culture model. We suggest that *GBA* mutations and reduced GCase activity may increase the risk for PD by inducing these same abnormalities in PD brain.

1. Introduction

Glucocerebrosidase 1 (GCase) is a ubiquitous lysosomal enzyme responsible for the breakdown of glucocerebroside to glucose and ceramide. Diverse mutations within the gene (*GBA*) that encodes GCase result in mutant enzymes with reduced activity and an autosomal recessive storage disorder (Gaucher disease – GD). GD patients have reduced GCase activity while heterozygote carriers generally have an intermediate level (Raghavan et al., 1980). GD is characterised by widespread accumulation of the GCase substrates glucocerebroside or glucosylsphingosine in many organs (Grabowski, 2008). Although *GBA* mutations cause a reduction in enzyme activity, this may not necessarily be the mechanism that mediates the pathogenesis of GD and alternative models include mis-trafficking of GCase and endoplasmic reticulum stress (Kov-Bar et al., 2011).

Alpha-synuclein positive Lewy bodies have been identified in the brains of GD patients and carriers who died with PD (Neumann et al., 2009; Wong et al., 2004). There are now persuasive data that *GBA* mutations are a major risk factor for PD and result in a clinical and pathological phenotype that is virtually indistinguishable from sporadic PD (Sidransky et al., 2009).

The mechanism(s) whereby *GBA* mutations increase the risk for PD remain unidentified. PD pathogenesis is thought to involve a number of pathways including mitochondrial dysfunction and oxidative stress (Schapira, 2006). Given the similar clinical and pathological phenotypes of *GBA*–PD and sporadic PD, we hypothesised that reduced GCase activity would result in biochemical events that would map to these same pathways considered of pathological relevance to familial and sporadic PD. We therefore investigated the effects of a specific GCase inhibitor (conduritol-β-epoxide) on mitochondrial function and free radical generation.

2. Materials and methods

2.1. Reagents

Reagents were supplied by Sigma–Aldrich (Poole, UK) and Merck (Nottingham, UK) unless otherwise stated.

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2.2. Cell cultures and treatments

SHSY-5Y cells were maintained as described (Alvarez-Erviti et al., 2010). During the course of continuous conduritol-β-epoxide (CβE; Universal Biologicals, Cambridge, UK) treatment, cells were split 1:3 every 3 days with fresh CβE added to a final concentration of 50 μM. GCase activity was monitored at different times throughout the course of the experiment to check that inhibition was maintained. Cell viability was checked by lactate dehydrogenase (LDH) release assays (Roche, UK). In brief, cells were plated into pairs of wells of a 48-well dish in phenol red-free media. After 48 h incubation of the control and CβE treatment, Triton X-100 was added to the medium of one of each pair of wells to a final concentration of 1% and the LDH level in the medium served as its total level. The LDH level found in the medium of the other well represents released LDH. Results were expressed as percentage LDH release from the total. For other assays, cells were harvested by trypsinisation. If required for assay of GCase, mitochondrial respiratory chain (MRC), or aconitase activities, cells were centrifuged at 1000 rpm, washed twice with phosphate-buffered saline (PBS) and stored as a pellet at −80 °C until assayed. Cells were then resuspended in 0.25 mM sucrose, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl) pH 7.4 and freeze–thawed three times prior to assay. If required for adenosine diphosphate (ADP) phosphorylation measurements, cell numbers were counted using haemocytometers (Immune Systems; Paignton, UK), and cells were centrifuged and resuspended in buffer for assays (see below).

2.3. Enzyme assays and ATP synthesis measurements

Respiratory chain (Schapira et al., 1990), proteasome (Schapira et al., 2006), aconitase (Bradley et al., 2000) and ADP phosphorylation activities (Korlipara et al., 2004) were measured by standard techniques as described previously. CβE-sensitive GCase activity (end-point measurement) was determined at 37 °C essentially as described (Prence et al., 1996) using 4-Methylumbelliferyl-β-D-glucopyranoside as substrate in a plate reader ('Synergy', LabTech; Brighton, UK). The increase in fluorescence of released 4-Methylumbelliferone at 460 nm following excitation at 360 nm was followed over 1 h. Protein levels were estimated using a bicinchoninic acid (BCA) kit (Pierce Thermo Fisher; Basingstoke, UK) with reference to the protein standard supplied with the kit.

2.4. Western blot analysis

Cells were harvested, washed with PBS and processed as described (Alvarez-Erviti et al., 2010). 25–40 μg of whole cell lysates were electrophoresed on Novex gels (NuPage 4–12%; Invitrogen, Paisley, UK) and transferred onto polyvinylidene fluoride membrane (Millipore; Watford, UK) and then probed with antibodies to porin (Merck; Darmstatd, Germany; 1/25000 dilution), adenine nucleotide transporter (ANT; Abcam, UK, 1/1000 dilution), microtubule-associated protein 1 light chain 3 (LC3, Clone D11; Cell Signalling, USA, 1/1000 dilution), alpha-synuclein (Becton–Dickinson, UK, 1/500 dilution), Glucocerebrosidase (GBA; Abcam, UK, 1.1000 dilution) or lysosome-associated membrane proteins (lamp1, clone H4A3; Abcam, UK, 1/1000 dilution) and all were normalised to β-actin (Abcam; 1/5000 dilution) Blots were developed using an enhanced chemiluminescence (ECL) kit (GE Healthcare; Little Chalfont, UK), exposed to X-ray film (GE Healthcare). The film was developed and signal intensities in the linear range were quantified by the 'Alphadigidoc' software package (AlphaInnotech; San Leandro, USA).

2.5. Live cell confocal imaging and analysis

Fluorescence of cells grown on 22 mm coverslips were measured by real-time confocal imaging as described (Gandhi et al., 2009; Duchen et al., 2003), using a Zeiss 510 laser scanning microscope equipped with an additional Enterprise UV laser source and a cooled charge coupled device camera, bathed in standard phenol red-free Hank's Buffered Salt Solution at room temperature. Mitochondrial membrane potential (Ψ_m) was quantified by steady-state fluorescence (excited using the 543 nm laser line and measured using a 560 nm longpass filter) of mitochondrial patterns produced by 25 nM tetramethyl rhodamine methyl ester (TMRM; Invitrogen) stained at room temperature for 45 min. Free radical generation was measured by the rate of ratiometric change of reduced and oxidised dihydroethidium (DHE; Invitrogen) fluorescence. 10 μM of DHE was loaded at room temperature, and measurements were made typically over 120 s. Oxidised DHE was measured with the 543 nm laser line and 560 nm long-pass filter, while for reduced DHE measurement it was excited at 351 and measured at 435–485 nm. Cells were treated accordingly and measured at the same time to minimise variability of fluorescence measurements. Individual cells were marked and mean fluorescence of individual cells measured by the ImageJ software (NIH, USA); where the image was captured with *Z*-stack, *z*-projection was performed using max intensity and net fluorescence was obtained by subtracting the background fluorescence. Mitochondrial morphology was measured on the circularity and aspect ratio from the TMRM images by ImageJ as described (Wang et al., 2011).

2.6. Creation of GBA knockdown SHSY-5Y stable cell lines

SHSY-5Y cells were transfected with a 'Hush' GBA knockdown plasmid (Origene, USA), empty plasmid and scrambled control (The sequence chosen for the *GBA* knockdown was: GTGTGTGTCTGCAATGCCACATACTGTGA). Stable clones were isolated following selection with puromycin (Sigma, UK) at 4 μg/ml and characterised by analysis of GCase activity, actin-normalised *GBA* mRNA by a 'StepOne' QPCR machine (Applied Biosystems, UK) using SyBr Green (Life Technologies, UK) and appropriate primers for *GBA* and β-actin (Eurofins, Germany) and GCase protein levels (by Western blotting). Clones were assessed after several passages (in the presence of a maintenance dose of 2 μg/ml puromycin) to check for the continuation of any knockdown effect.

2.7. Statistical analysis

Where multiple comparisons were made, one-way ANOVA tests were performed followed by Dunnett post test analysis in order to determine statistical significance. Student's *t*-tests were used for comparing statistical significance between 2 populations. A p value of < 0.05 was considered as significantly different.

3. Results

3.1. CβE

CβE has been reported to be a selective inhibitor of GCase activity (Prence et al., 1996; Newburg et al., 1986) and we have confirmed in SHSY-5Y cells that 50 μM CβE decreased GCase activity to $\leqslant$5% of untreated cells and maintained the inhibition of GCase activity over 30 days (Suppl. Fig. 1). This concentration of CβE has also been previously reported to result in a greater than 2-fold increase of glucocerebroside over 24 days (Prence et al., 1996). In our experiments, 30 days CβE treatment had no effect on cell viability as judged by LDH release (Suppl. Fig. 2).