Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Inhibition of LRRK2 kinase activity stimulates macroautophagy ☆

Claudia Manzoni [a,*], Adamantios Mamais [a,b], Sybille Dihanich [a], Rosella Abeti [a], Marc P.M. Soutar [a], Helene Plun-Favreau [a], Paola Giunti [a], Sharon Tooze [c], Rina Bandopadhyay [a,b], Patrick A. Lewis [a,d,**]

[a] Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London, WC1N 3BG, UK
[b] Reta Lila Weston Institute and Queen Square Brain Bank, UCL Institute of Neurology, Queen Square, London, WC1N 3BG, UK
[c] London Research Institute, Cancer Research UK, Lincoln's Inn Fields, London, EC1V 4AD, UK
[d] School of Pharmacy, University of Reading, Whiteknights, Reading, RG6 6AP, UK

ARTICLE INFO

Article history:
Received 14 January 2013
Received in revised form 19 July 2013
Accepted 23 July 2013
Available online xxxx

Keywords:
LRRK2
Macroautophagy
Parkinson's disease
LC3
p62
WIPI2

ABSTRACT

Leucine Rich Repeat Kinase 2 (LRRK2) is one of the most important genetic contributors to Parkinson's disease. LRRK2 has been implicated in a number of cellular processes, including macroautophagy. To test whether LRRK2 has a role in regulating autophagy, a specific inhibitor of the kinase activity of LRRK2 was applied to human neuroglioma cells and downstream readouts of autophagy examined. The resulting data demonstrate that inhibition of LRRK2 kinase activity stimulates macroautophagy in the absence of any alteration in the translational targets of mTORC1, suggesting that LRRK2 regulates autophagic vesicle formation independent of canonical mTORC1 signaling. This study represents the first pharmacological dissection of the role LRRK2 plays in the autophagy/lysosomal pathway, emphasizing the importance of this pathway as a marker for LRRK2 physiological function. Moreover it highlights the need to dissect autophagy and lysosomal activities in the context of LRRK2 related pathologies with the final aim of understanding their aetiology and identifying specific target for disease modifying therapies in patients.

1. Introduction

Leucine Rich Repeat Kinase 2 (LRRK2) is a multidomain protein of unknown function containing two enzymatic domains, a GTPase (Ras of Complex Proteins, ROC) and a kinase, and several protein/protein interaction domains [1]. LRRK2 has been implicated in a number of cellular processes, including the control of neurite branching, synaptic vesicle recycling, macroautophagy (hereafter referred to as autophagy), protein synthesis through the mammalian target of rapamycin (mTOR) pathway and mitochondrial homeostasis [2]. The physiological function of LRRK2 in the regulation of these processes is, however, unclear.

The central role of this protein in Parkinson's disease (PD) has been highlighted by the discovery of autosomal dominant mutations in LRRK2 causing familial Parkinson's disease and the subsequent identification of the *LRRK2* locus as a risk factor for sporadic disease [3,4]. A key question regarding the role of autosomal dominant coding change mutations in PD is what the cellular consequences of these mutations are, and how they lead to disease [2]. Penetrant coding mutations are found exclusively in the enzymatic core of LRRK2 — the ROC/COR/kinase triptych [4], leading to a number of studies examining the impact of mutations on the enzymatic activities of this protein. The G2019S mutation, the most common disease linked variant in LRRK2, has been consistently associated with increased kinase activity, and mutations in the ROC and COR domains display reduced GTPase activity [5–9]. However, thus far no biochemical phenotype has been consistently linked to mutations in all three of these domains. The only reported cellular phenotype that consistently correlates with penetrant mutations is cytotoxicity, which is dependent upon kinase activity [10–12].

A number of recent reports have suggested a role for LRRK2 in the autophagy/lysosomal pathway [13–21]. Data from a range of cell lines and patient derived cells have revealed alterations in key markers of autophagy in the presence of mutations in LRRK2, although the precise point in the pathway that links LRRK2 to this process has not been identified [13,14,18–20]. The relationship between LRRK2 and autophagy has been further highlighted by studies in animal models lacking LRRK2 or expressing a mutant form of the protein [15,16,21]. Knockdown studies support a complicated link between LRRK2 and the induction/regulation of autophagy, in particular the demonstration

Abbreviations: LRRK2, Leucine Rich Repeat Kinase 2; mTOR, Mammalian target of rapamycin; ROC, Ras of Complex Proteins; COR, C-terminal of ROC domain; SDS, Sodium dodecyl sulphate; EDTA, Ethylene di-ammonium tetra acetic acid; DPBS, Dulbecco's phosphate buffered saline; DMSO, Dimethylsulfoxide

* Corresponding author.
** Correspondence to: P.A. Lewis, Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London, WC1N 3BG, UK.
E-mail addresses: c.manzoni@ucl.ac.uk (C. Manzoni), patrick.lewis@ucl.ac.uk (P.A. Lewis).

0167-4889/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.07.020

that loss of LRRK2 results in biphasic changes in autophagy over the course of mouse development [21]. Data from fly models of LRRK2 dysfunction have suggested that LRRK2 may function in the mTOR pathway, implicating LRRK2 in a pathway with an important role in regulating autophagy, although these data have proved controversial [22,23]. Intriguingly, LRRK2 has also been identified as a risk factor in a number of human diseases characterized by a strong pathogenic link to autophagy (in addition to PD): Crohn's disease, cancer and leprosy [24–26]. A key research challenge in LRRK2 biology is, therefore, to elucidate the precise role of this protein in autophagy.

To clarify the role of LRRK2 in the regulation of autophagy, this study takes advantage of recently described inhibitors of LRRK2 kinase activity [27,28] to test whether the kinase activity of endogenous LRRK2 is important for this pathway at a cellular level, and to delineate the point at which LRRK2 intervenes in autophagy.

2. Materials and methods

2.1. Inhibitors

The LRRK2-in1 and the CZC-25146 compounds were purchased from the Department of Biochemistry, University of Dundee, UK. GSK 2578215A was purchased from Tocris Bioscience. Bafilomycin A1 (B1793-2UG) and cyclohexamide (01810-1G) were purchased from Sigma-Aldrich.

2.2. Antibodies

Antibodies used were as follows: rabbit LC3 antibody (NB100-2220, Novus Biologicals); mouse LC3 antibody (5F10, Nanotools), LRRK2 antibodies (N138/6, NeuroMab and 3514-1, Epitomics); total S6 antibody (2317, Cell Signalling); phospho Ser235/236S6 antibody (2211S, Cell Signalling); total P70S6K antibody (sc-8418, Santa Cruz); phospho Thr389 P70S6K (sc-11759, Santa Cruz); total 4EBP1 (81149, Santa Cruz); phospho Ser65 4EBP1 (9451S, Cell Signaling); mouse p62 antibody (610833, BD Transduction Labs); rabbit p62 antibody (BML-PW9860-0025, Enzo Life Sciences); mouse WIPI2 antibody (kindly supplied by Prof. S. Tooze) and mouse β-actin antibody (A1978, Sigma Aldrich). LRRK2 phosphorylation was assessed using rabbit phospho Ser935-LRRK2 (5099-1, Epitomics).

2.3. Cell culture, cell treatments

Cell lines were grown in DMEM containing 10% FCS, with the exception of the mTOR stimulation experiment as described below. Human neuroglioma H4 cells (ATCC number HTB-148), human neuroblastoma SHSY5Y (ATCC number CRL-2266) or Human Embryonic Kidney (HEK) cells (ATCC number CRL-1573) were seeded at a concentration of 2×10^5 cell/ml in 6 wells plates (2 ml for each well). After 6 hours from plating, cells were treated with LRRK2 inhibitors LRRK2-in1, CZC-25146 and GSK 2578215A. The concentrations of inhibitors, as used during the treatment, are reported in every experiment shown in the text. All compounds were dissolved in DMSO. For each experiment, DMSO vehicle controls were added. Cells were incubated overnight with LRRK2 kinase inhibitors and fresh treatment was replaced the following morning for 2.5 hours before cell lysis.

Cells were then washed once in Dulbecco's phosphate buffered saline (DPBS) and collected in a lysis buffer containing: 0.5% Triton X-100, 2 mM ethylene di-ammonium tetra acetic acid (EDTA), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), protease inhibitors (cOmplete, protease inhibitor cocktail, Roche) and phosphatase inhibitors (Halt phosphatase inhibitor cocktail, Pierce) in 50 mM TRIS-HCl pH 7.5.

For mTOR pathway experiments, cells were seeded as described above; mTOR inhibition was achieved by overnight (16 hours) serum deprivation followed by substitution of the growing medium with Earle's balanced salts solution for 2 hours. Re-activation of the mTOR pathway was obtained after starvation by feeding cells with MEM non-essential amino acid supplement added directly to the Earle's solution for 30 minutes. Non-starved, starved and amino acid fed cells were then washed once in DPBS and collected in lysis buffer.

2.4. Immunoblotting

Cell lysates were frozen immediately upon collection; following thawing, they were clarified by centrifugation at 10,000*g* for 5 minutes at 4 °C prior to use. Lysate protein concentrations were assessed by BCA assay (BCA Protein Assay Kit, Pierce) and samples containing 10 μg of proteins were prepared for SDS-PAGE with the addition of NuPAGE sample buffer (Invitrogen), and denatured for 10 minutes at 70 °C. Electrophoresis was performed using NuPAGE, Novex precasted Bis-Tris 4–12% gels (Invitrogen), according to the manufacturer's instructions. After electrophoresis, proteins were transferred to 0.45 μm PVDF membranes (IPVH00010, Immobilon Millipore) for 2 hours. Proteins were identified by the appropriate primary and secondary HRP antibodies and visualized using Enhanced Chemiluminescence (ECL) and X-Ray films (Super RX, medical X-Ray film, Fujifilm). Films were acquired as images in jpg format using an EPSON Perfection 4870 photo scanner and processed by the ImageJ software (http://rsbweb.nih.gov/ij/) to quantify area and total intensity of each single band. Statistical analyses were performed by Prism software (GraphPad) as described in the text.

2.5. Generation of shRNA scramble control/LRRK2 knockdown (KD) stable H4 cell line

H4 cells were transfected with 2 μg LRRK2 shRNA or scramble shRNA (V3LHS-644167, Thermo Fisher Scientific) using Effectene (Qiagen) transfection reagent according to the manufacturer's instructions. ShRNA vectors contain a puromycin resistance gene therefore 48 hours post transfection cells were treated with 2 μg/ml puromycin supplemented DMEM. Media were changed every 2 days (removal of dead cells) for 2 weeks in order to select for puromycin-resistant cells containing shRNA.

2.6. Neutral red staining

Cells were treated with DMSO, LRRK2-in1 (1 μM over night) or with the autophagy inhibitor bafilomycin (40 nM, over night); at the end of the treatment, the cell culture medium was supplemented with a solution of 3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride (Neutral red, Sigma Aldrich) with a final concentration of 80 ng/ml, for 30 minutes [29]. Cells were washed twice with DPBS and dissolved in a destaining solution composed of 50% ethanol, 49% deionized water, 1% glacial acetic acid and the absorbance was recorded by the use of a multiwell plate reader at the wavelength of 540 nm. For every 96 well plates used in the assay, a replicate of 2 wells per single column was used to determine protein concentration (BCA assay). Data were expressed as absorbance at 540 nm normalized to the absorbance recorded for the BCA assay for every single column within the plate. The final results in the graph were expressed as percentage of Neutral red staining in comparison with untreated controls.

2.7. Cytotoxicity

Cells were treated with LRRK2 kinase inhibitors as described above; at the end of the treatment, the cell culture medium was added of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich) to the final concentration of 500 μg/ml for 3 hours. Cell medium was then discarded and the formazan crystals accumulated within the energetically active cells were dissolved in 100% DMSO and the absorbance measured using a multiwell plate reader at 570 nm.

Please cite this article as: C. Manzoni, et al., Inhibition of LRRK2 kinase activity stimulates macroautophagy, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamcr.2013.07.020