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Increased fatty acid synthesis inhibits nitrogen starvation-induced autophagy in lipid droplet-deficient yeast

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ABSTRACT

Macroautophagy is a degradative pathway whereby cells encapsulate and degrade cytoplasmic material within endogenously-built membranes. Previous studies have suggested that autophagosome membranes originate from lipid droplets. However, it was recently shown that rapamycin could induce autophagy in cells lacking these organelles. Here we show that lipid droplet-deprived cells are unable to perform autophagy in response to nitrogen-starvation because of an accelerated lipid synthesis that is not observed with rapamycin. Using cerulenin, a potent inhibitor of fatty acid synthase, and exogenous addition of palmitic acid we could restore nitrogen-starvation induced autophagy in the absence of lipid droplets.

1. Introduction

Macroautophagy, hereafter referred to as “autophagy”, is a degradative pathway widely conserved in eukaryotes from yeast to mammals [1]. It is induced by various physiological stresses, such as starvation, and allows degradation of cytoplasmic materials and organelles to create novel building blocks to adapt a changing environment [2]. Defects in this process have been identified in the pathology of several diseases including cancer, some neurodegenerative diseases and myopathies [3–6]. Autophagy begins with the expansion of a cup-shaped membrane structure called the isolation membrane, or phagophore, which encapsulates the cytoplasmic materials or organelles that are to be degraded, while expanding. The phagophore eventually closes to form a double membrane structure called the autophagosome [7]. Its content is then delivered to the vacuole for degradation.

To date, 38 autophagy-related genes have been identified in the yeast *Saccharomyces cerevisiae* [8]. Many of them are shared by the Cytoplasmic to Vacuole (CVT) pathway that constitutively addresses proteins to the vacuole [9] and others are required for selective autophagy processes. A possible contribution of lipid droplets to autophagy has been reported [10–12]. Lipid droplet is a conserved structure found in most eukaryotic cells. It consists in a core of neutral lipids, mainly triacylglycerol and steryl-esters, which are surrounded by a phospholipid monolayer. In *S. cerevisiae*, lipid droplets biogenesis is strictly controlled by the enzymes necessary for neutral lipid synthesis [13]. These comprise two diacylglycerol acyl transferases encoded by *DGA1* and *LRO1* which are responsible for TAG biosynthesis [14] whereas steryl-esters are synthesized by the action of the *ARE1* and *ARE2* gene products [15,16]. Thus, yeast mutants deleted for these 4 genes do not produce detectable neutral lipids and are completely devoid of

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; HA, hemagglutinin; TOR, target of rapamycin; YNB, yeast nitrogen base; YPD, yeast extract/peptone/dextrose.

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lipid droplets [13]. Two studies in yeast have shown that such mutants are incapable of autophagy in response to nitrogen starvation [10,12]. In mammals too, lipid droplets have been implicated in the regulation of autophagy [11]. From these studies it was suggested that lipid droplet contribution to autophagy would consist in providing fatty acids and lipids necessary for membrane autophagosome biogenesis [17]. Recently, this view was challenged by the observation that lipid droplet-deficient yeast is still capable to induce autophagy in response to rapamycin [18]. Moreover the authors showed that in the absence of lipid droplets, nitrogen starvation-induced autophagy defects could be corrected by inhibiting fatty acid biosynthesis and altering phospholipid composition. This observation was somewhat unexpected since it is known that inhibition of fatty acid synthesis in a wild type strain strongly impairs autophagy [10]. In the present paper, we come to a similar conclusion as Velazquez et al. (2016) through a different experimental approach. Firstly, we establish that nitrogen starvation, but not rapamycin treatment, triggers an increase in lipid biosynthesis. Then we show that nitrogen starvation-induced autophagy can be restored in lipid droplet-deficient yeast by a controlled supply of fatty acid. Finally, we correlate the defects in autophagy with electron microscopic evidence of tangled ER membranes and we show that our strategy of a controlled fatty acid supply to restore autophagy also leads to the disappearance of these aberrant membrane tangles.

2. Materials and methods

2.1. Reagents, antibodies, and plasmids

Rapamycin (Santa-Cruz) and cerulenin (SIGMA) were solubilized in DMSO and added at a final concentration of 200 nM (0.2 μg/ml) and 40 μM (10 μg/ml) respectively unless otherwise stated. The vacuole labeling dye, FM4-64 (Santa Cruz) was dissolved in DMSO and used at 20 μM. Palmitic acid was dissolved in DMSO and used at a final concentration of 200 μM.

Mouse antibodies directed against GFP were obtained from Roche; mouse anti-Pgk1 antibodies and mouse anti HA antibodies were purchased from Invitrogen and SIGMA respectively. HRP conjugated anti-mouse secondary antibodies were from SIGMA.

For GFP–Atg8 processing, the plasmid pRS416 expressing GFP–Atg8 was used [19]. Plasmid pPHY2427 encoding 3xHA-Atg13 under the control of the *CUP1* promoter in pRS426 was kindly provided by Pr. Hermann [20].

2.2. Yeast strains, media and growth conditions

All yeast strains described in this study are derived from BY (Euroscarf). BY4742 was used as the wild-type strain. RS4Δ (BY4742; *MATα are1::kanMX4 are2::kanMX4 lro1::TRP1 dga1::Lox-HIS3-Lox ura3 leu2 lys2*) was kindly provided by Roger Schneiter. Yeast strains were cultured in minimal YNB medium (yeast nitrogen base with ammonium sulfate and 2% glucose) supplemented with the appropriate amino acids and bases, or complete YPD medium (1% yeast extract, 1% peptone, 2% dextrose). For nitrogen starvation, unless otherwise stated, overnight cultures in YNB medium were inoculated in YPD medium and propagated for at least two generations; cells were harvested by centrifugation, washed with distilled water and autophagy was induced by inoculation in YNB medium without ammonium sulfate and amino acid (-N). Autophagy induction with rapamycin was achieved by adding rapamycin into YPD medium.

2.3. Immunoblotting analysis

Approximately 10^8 cells were harvested, and lysed by alkaline whole cell extraction (0.2 M NaOH; 0.3% β-mercaptoethanol) followed by TCA precipitation. Protein extracts were subsequently washed with acetone, dried and suspended in 100 μl of 5% (w/v) SDS. Finally, 100 μl of Laemmli buffer (20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.04% Bromophenol Blue, 0.0625 M Tris-HCl pH 6.8) was added. Samples were incubated 10 min at 65 °C prior to loading on a 12% polyacrylamide gel. Routinely, lysates from the equivalent of 4×10^6 of cells were subjected to immunoblot analysis. After western blotting onto nitrocellulose and blocking the membrane with 5% non-fat milk, 0.1% Tween 20 in Tris-buffered saline, the blotted proteins were probed with mouse anti-GFP antibodies (1/2000) or with mouse anti-Pgk1 antibodies (1/10000) or mouse anti HA antibodies (1/2000). HRP conjugated anti-mouse secondary antibody (SIGMA) was used at 1/1000. Detection was performed with ECL prime from Amersham.

2.4. Fluorescence microscopy

Microscopy was performed on a FV1000 Olympus confocal microscope using a 60× water immersion lens (NA 1.20) coupled to a 2.3× numerical zoom (0.12 μm per pixel). The excitation wavelengths for GFP or FM4-64 were set to 488 nm and 543 nm respectively. Image acquisition and conversion were performed separately for green (520 nm) and red (603 nm) channels and processed with the Olympus Fluoview version 4.1 software. Transmission images were acquired with differential interference contrast optics.

2.5. Pulse labeling, extraction and analysis of lipids

An overnight culture in YPD medium at 27 °C was propagated to OD~1 (2×10^7 cells/ml). The culture was divided into three fractions. The first fraction was immediately used for lipid labeling (T0 sample). Cells from the other two fractions were washed with distilled water and resuspended with rapamycin-containing YPD (R sample) or nitrogen free medium (-N sample) and incubated for 2 h, before lipid labeling. Lipid labeling was performed in triplicate for 15 min at 27 °C, after addition of 2 μCi/ml [2-^{14}C]-acetic acid, sodium salt (1994 MBq/mmol; PerkinElmer, Courtaboeuf, France). Acetate incorporation was stopped by addition of 20 mM NaN_3. Cells were pelletted and stored at −20 °C.

For total lipid extraction, the cell pellet was resuspended with 1 ml of 10 mM Trs HCl pH7.5, 40 mM β-mercaptoethanol and incubated for 5 min at room temperature. The cells were then centrifuged and resuspended with 600 μl of lysis buffer (20 mM Tris HCl pH7.5, 150 μg of lyticase 250 kU) and incubated for 30 min at 27 °C.

Lipids were extracted using methanol-chloroform (2:1) [21], dried and counted.

2.6. Electron microscopy

Cells were collected after 2 h of treatment and processed for electron microscopy as previously described [22] and observed on JEM1010 JEOL electron microscope.

3. Results and discussion

3.1. Lipid droplet-deficient yeast activates autophagy in response to rapamycin

The recent observation that autophagy induced by nitrogen

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