



Cycloheximide inhibits starvation-induced autophagy through mTORC1 activation



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ABSTRACT

Protein synthesis inhibitors such as cycloheximide (CHX) are known to suppress protein degradation including autophagy. The fact that CHX inhibits autophagy has been generally interpreted to indicate that newly synthesized protein is indispensable for autophagy. However, CHX is also known to increase the intracellular level of amino acids and activate mTORC1 activity, a master negative regulator of autophagy. Accordingly, CHX can affect autophagic activity through inhibition of *de novo* protein synthesis and/or modulation of mTORC1 signaling. In this study, we investigated the effects of CHX on autophagy using specific autophagy markers. We found that CHX inhibited starvation-induced autophagy but not Torin1-induced autophagy. CHX also suppressed starvation-induced puncta formation of GFP-ULK1, an early-step marker of the autophagic process which is regulated by mTORC1. CHX activated mTORC1 even under autophagy-inducible starvation conditions. Finally, the inhibitory effect of CHX on starvation-induced autophagy was cancelled by the mTOR inhibitor Torin1. These results suggest that CHX inhibits starvation-induced autophagy through mTORC1 activation and also that autophagy does not require new protein synthesis at least in the acute phase of starvation.

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1. Introduction

Macroautophagy, hereafter referred to simply as autophagy, is a major intracellular degradation system [1–3]. Cytoplasmic materials are engulfed by a double-membrane autophagosome and delivered to the lysosome, resulting in degradation of the enclosed contents together with the inner autophagosomal membrane. Autophagy is typically induced by starvation and is thought to be mainly regulated by post-translational modifications of autophagy-related proteins and lipid modifications [4,5], although recent studies also suggest involvement of transcriptional regulation [6–12].

Early studies demonstrated that inhibitors of protein synthesis such as cycloheximide (CHX) also inhibits starvation-induced protein degradation [13–16]. More recently, the specific effects of CHX on autophagy were tested and controversial results were obtained. In budding yeast, Takeshige et al. showed that starva-

tion-induced accumulation of autophagic bodies in the vacuole was strongly suppressed by CHX treatment [17]. By contrast, Abeliovich et al. reported that pretreatment of CHX did not affect induction of autophagy by rapamycin, although the size of autophagosomes became smaller [18]. In mammalian cells, CHX was shown to inhibit autophagosome formation [19,20]. However, Lawrence et al. reported that pretreatment with CHX for 3 h inhibited autophagosome-lysosome fusion without affecting autophagy induction [21]. These studies showed that CHX inhibits autophagy, although the step affected seems to be different among studies. Most of the studies suggested that newly synthesized protein is required for autophagy. On the other hand, it was reported that CHX has no inhibitory effect on glucagon-induced autophagy [22] or virus infection-induced autophagy [23].

It should be noted that inhibition of protein synthesis can reduce consumption of amino acids and thereby increase the level of intracellular amino acids [13,24,25]. It is also known that CHX activates mTORC1, a master negative regulator of autophagy, through accumulation of intracellular amino acids following inhibition of protein synthesis [24,26]. Therefore, CHX can affect autophagic activity through inhibition of *de novo* protein synthesis and/or modulation of mTORC1 signaling.

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In this study, using specific markers, we investigated whether CHX indeed inhibits autophagy, and if so, at which step autophagy is blocked.

2. Methods

2.1. Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 Units/ml penicillin, and 50 µg/ml streptomycin (regular medium) in a 5% CO₂ incubator. MEFs stably expressing GFP-fused microtubule-associated protein 1 light chain 3 (GFP-LC3) or GFP-ULK1 were generated previously [27]. For amino acid starvation treatment, cells were washed with phosphate-buffered saline (PBS) and incubated in amino acid-free DMEM with 10% dialyzed FBS. Serum was dialyzed against PBS (6 L) for 24 h using Slide-A-Lyzer 3500 MWCO dialysis cassette (Thermo Scientific).

2.2. Antibodies and reagents

Antibodies against 4E-BP1 (#9452), S6 kinase 1 (S6K1) (#9202), S6K1 pT389 (#9234), general amino acid control non-repressible 2 (GCN2) (#3302), Akt (#9272), and Akt pT308 (#9275) were purchased from Cell Signaling Technology. Antibody against GCN2 pT899 (ab75836) was purchased from Abcam. Mouse monoclonal anti-β-actin antibody was purchased from Sigma–Aldrich (A2228). Anti-LC3 rabbit polyclonal antibody was previously generated [28]. CHX and chloroquine (CQ) were purchased from Sigma–Aldrich.

2.3. Fluorescence microscopy of GFP-LC3 punctate structures

Cells grown on coverslips were washed with PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min. Cells were observed with a fluorescence microscope (IX81; Olympus) or a confocal laser microscope (FV1000D; Olympus) using a 60x PlanApoN oil immersion lens (1.42 NA; Olympus). For final output, images were processed using Photoshop 7.0.1 software (Adobe Systems). The number of punctate structures was determined as follows: punctate structures were extracted using the top hat operation (parameter: 100 × 100 pixel area), and a binary image was created. Small structures (less than 3 × 3 pixel area) were removed using an open operation. The puncta were counted using the integrated morphometry analysis program. False dots were removed by comparison with the original image.

2.4. GFP-ULK1 immunohistochemistry

MEFs expressing GFP-ULK1 grown on coverslips were washed with PBS and fixed in 4% PFA in PBS for 10 min. Fixed cell were permeabilized with 50 µg/ml digitonin in PBS for 5 min, blocked with 3% bovine serum albumin in PBS for 30 min, and incubated with anti-GFP antibody for 1 h. After washing, cells were incubated with Alexa-Fluor-488-conjugated goat anti-rat IgG secondary antibodies (Invitrogen) for 1 h.

2.5. Western blot and quantification

Cell lysates were prepared in a regular lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.4 mM Na₃VO₄, 10 mM sodium pyrophosphate, and Complete EDTA-free protease inhibitor (Roche Applied Science)). The lysates were clarified by centrifugation at 17,400g for 5 min

and then boiled in sample buffer. Samples were separated by SDS–PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Immunoblot analysis was performed and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce) or Immobilon Western (Millipore). The signal intensities were analyzed using an LAS-3000mini imaging analyzer and Multi Gauge software version 3.0 (Fujifilm). Contrast and brightness adjustment was applied uniformly to the images using Photoshop 7.0.1.

2.6. Statistical analyses

Data were expressed as mean ± SE. Unpaired Student *t* test and analysis of variance (ANOVA) was used to determine differences between two and three groups, respectively.

3. Results

3.1. Translation inhibitors block starvation-induced autophagy but not Torin1-induced autophagy

We first determined whether CHX inhibits autophagy by monitoring the modification of LC3 [29,30]. LC3 is one of the mammalian homologs of autophagy-related 8 (Atg8) and is usually present in the cytosol in its LC3-I form. When autophagy is induced, LC3 localizes on the autophagosomal membrane in its lipid-conjugated form LC3-II. When autophagy was induced by withdrawal of amino acids or treatment with the mTOR inhibitor Torin1, the amount of LC3-II was increased (Fig. 1A). However, when cells were pretreated with 50 µg/ml CHX for 5 min, starvation-induced LC3-II formation was suppressed (Fig. 1A). By contrast, pretreatment of CHX did not inhibit Torin1-induced LC3-II formation. These observations are consistent with findings from previous studies in yeast that CHX inhibits autophagy induced by starvation [17] but not by rapamycin [18]. Similar results came from cells treated with puromycin, another translation inhibitor (Fig. 1A).

Inhibition of autophagy by CHX was further confirmed by fluorescence microscopy of MEFs stably expressing GFP-LC3. Amino acid starvation or Torin1 treatment induced formation of GFP-LC3 punctate structures (Fig. 1B), which represented autophagosomes/autolysosomes [29,30]. However, the induction of GFP-LC3 puncta by starvation but not by Torin1 treatment was inhibited by pretreatment with CHX. These data suggest that blockage of protein translation inhibits starvation-induced autophagy but not Torin1-induced autophagy.

3.2. CHX restores the mTORC1 activity under autophagy-inducible starvation conditions

It has been reported that CHX can activate mTORC1 by accumulation of intracellular amino acids [24]. Therefore, we measured the mTORC1 activity based on phosphorylation status of its substrates S6K and 4E-BP1 under autophagy-inducible conditions. Torin1 treatment caused strong dephosphorylation of S6K and 4E-BP1 regardless of CHX treatment, suggesting that mTORC1 was inhibited (Fig. 1A). Upon amino acid starvation, mTORC1 activity was also inhibited. However, when cells were pretreated with CHX, phosphorylation of both S6K and 4E-BP1 was maintained even after 1-h amino acid-starvation treatment as previously reported (Fig. 1A) [24]. These data suggest that the inhibitory effect of CHX on autophagy could be due to modulation of mTORC1 activity rather than inhibition of protein synthesis.

Next we tested whether CHX can reactivate mTORC1 and suppress autophagy in cells that had been starved. MEFs expressing GFP-LC3 were deprived of amino acids for 45 min, and then treated

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