Analytical Biochemistry 530 (2017) 57–67

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Quantification of autophagy flux using LC3 ELISA

Sung-hee Oh [a], Yong-bok Choi [b], June-hyun Kim [b], Conrad C. Weihl [c], Jeong-sun Ju [a, *]

[a] Department of Exercise Science, Research Institute of Sports Science, The University of Suwon, 17 Wauan-gil, Bongdam-eup, Hwaseong-si, Gyeonggi-do, 18322, South Korea
[b] Department of Biotechnology and Bioscience, School of Bioindustry, The University of Suwon, 17 Wauan-gil, Bongdam-eup, Hwaseong-si, Gyeonggi-do, 18322, South Korea
[c] Department of Neurology, Washington University School of Medicine, PO Box 8111, 660 South Euclid Avenue, St Louis, MO 63110, USA

ARTICLE INFO

Article history:
Received 1 February 2017
Received in revised form
18 April 2017
Accepted 1 May 2017
Available online 4 May 2017

Keywords:
Autophagy
Mitophagy
ELISA
LC3 turnover
Skeletal muscle

ABSTRACT

Macroautophagy (hereafter referred to as autophagy) is a degradation system that delivers cytoplasmic materials to lysosomes via autophagosomes. Autophagic flux is defined as a measure of autophagic degradation activity. Despite several methods for monitoring autophagic flux being currently utilized, interest in finding a highly accurate, sensitive and well-quantifiable assay is still growing. Therefore, we introduce a new approach analyzing autophagic flux *in vitro* and *in vivo* using enzyme-linked immunosorbent assay (ELISA) technique. In order to adapt this assay from LC3-II turnover measured by Western blot in the presence and absence of lysosomal inhibitors, we induced autophagy by starvation or rapamycin and mitophagy (mitochondrial degradation by autophagy) by CCCP in C2C12 myotubes for 8 h and in mice for 48 h with and without Bafilomycin A1 or colchicine treatment, respectively. Following subcellular fractionation of mouse skeletal muscle cells and tissue, cytosolic, membrane, and mitochondrial fractions were analyzed through a sandwich ELISA using two LC3 antibodies, LC3 capture and HRP-conjugated LC3 detection antibodies. Using this ELISA, changes in the membrane-bound or mitochondrion-associated LC3-II levels, and the ratio of the LC3-II from each fraction to LC3-I levels (cytosolic fraction) were evaluated for measuring autophagy and mitophagy flux. This study demonstrates that this ELISA was more sensitive and reliable to measure autophagic/mitophagic flux in both *in vitro* and *in vivo*, compared with the most commonly used LC3 turnover assay via Western blot.

Introduction

Autophagy is an evolutionarily conserved intracellular catabolic process that is used by all cells to degrade dysfunctional or unnecessary cytoplasmic components through delivery to the lysosome [1]. This process constitutes an intracellular quality control mechanism as well as a response to a wide range of stimuli, such as nutrient deprivation, hypoxia, reactive oxygen species, protein aggregates, damaged organelles, and drugs (e.g. rapamycin). The activation of autophagy by these stimuli involves multiple signaling pathways. For example, the mechanistic target of rapamycin (mTOR), a serine/threonine kinase, negatively regulates the activation of autophagy. Autophagy is regulated by the Atg family of proteins, which participate in all stages of the autophagy process: induction, autophagosome formation, lysosomal fusion, and autolysosome formation, and degradation [2]. Autophagy begins with the formation of autophagosomes, which can be determined by visualization of the LC3-II protein because of its localization on the autophagosome membrane. LC3-II derives from a proLC3 30 kDa protein after cleavage by Atg4 to produce the active cytosolic form LC3-I. This in turn is activated by Atg7, and then transferred to Atg3, a second E2-like enzyme, becoming a membrane-bound form, LC3-II, which is degraded by autophagy [3]. LC3-II protein levels serve as a read-out of an autophagosome number, and measuring the conversion of LC3-I to LC3-II by immunoblot is indicative of autophagic activity [4]. However, limitations to the interpretation of LC3-II levels through immunoblot were previously emphasized and an assay for autophagy flux monitoring LC3 turnover has been proposed: LC3-II levels should only be evaluated in the presence and absence of inhibitors which blocks autophagosome and hence LC3-II degradation [4,5]. Autophagic flux, which encompasses the entire process of autophagy, is a more reliable indicator of autophagic

* Corresponding author. Department of Exercise Science, Research Institute of Sports Science, The University of Suwon, 17 Wauan-gil, Bongdam-eup, Hwaseong-si, Gyeonggi-do, 445-743, South Korea.
E-mail address: jju625@hotmail.com (J.-s. Ju).

http://dx.doi.org/10.1016/j.ab.2017.05.003
0003-2697/

activity [6]. This approach has been well described in an *in vitro* model [7], and in an *in vivo* system [8], and currently this method is one of the most widely and routinely used to monitor autophagic flux. However, when analyzing LC3 by Western blotting, frequently, it is a little challenging for the precise quantification of two LC3 isoforms, simultaneously, and the pattern of LC3-I to LC3-II conversion does not always occur in a predictable manner. This may be due to cell-specific differences in the amounts of LC3-I versus LC3-II or in transcriptional regulation of LC3 by different kinds of stress or stimuli [6]. For example, LC3-I is very abundant in brain tissue, and the intensity of the LC3-I band may obscure detection of LC3-II. Therefore, it was suggested that, in brain tissue, immunoblot analysis of the membrane and cytosol fraction from a cell lysate, upon appropriate loading of samples to achieve quantifiable and comparative signals, can be useful to measure LC3 isoforms [6]. Besides that, Western blotting can often be a frustration due to its unreliable results and it is a very delicate process requiring the correct amounts of each component and optimized the experimental conditions (i.e. gel concentration, a balance in total protein, primary/secondary antibody concentrations, and exposure time, etc.) for successful protein quantification. To reduce these technical problems and develop a simpler and more sensitive method than Western blotting for monitoring LC3 turnover *in vitro* and *in vivo*, we applied a similar immunodetection method, ELISA technique, for measuring autophagy flux using LC3 antibodies.

In order to adapt this ELISA to measure autophagy flux *in vitro* and *in vivo*, we induced autophagy by starvation or rapamycin, and mitophagy by carbonyl cyanide m-chlorophenylhydrozone (CCCP, a mitochondrial uncoupler), a well-known inducer of mitophagy[6] in C2C12 cells for 8 h and in mice for 48 h with and without the administration of bafilomycin A1 or colchicine, respectively. Using a protocol for the subcellular fractionation of mouse skeletal muscle cells and tissue, the cytosolic, membrane, and mitochondrial fractions were obtained and analyzed through a sandwich ELISA using two LC3 antibodies: LC3 capture and HRP-conjugated LC3 detection antibodies. In the present study, we describe the determination of both autophagy and mitophagy flux via the LC3 sandwich ELISA technique using subcellular fractions of skeletal muscle cells and tissue.

Materials and methods

Materials and antibodies

BafilomycinA1 (B1793), CCCP (C2759), colchicine (C9754), anti-LC3B polyclonal (L7543) and anti-SERCA2 (MAB2636) antibodies were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Rapamycin (R-5000) was purchased from LC Laboratories (www.LCLabs.com). Anti-phospho-S6 (ser235/236) (2211) and anti-S6 ribosomal protein (2217) antibodies were from Cell Signaling Technology (www.cellsignal.com). Anti-citrate synthase (16131-1-AP) polyclonal antibody was from proteintech (www.ptglab.com). Anti-GPADH (ADI-CSA-335-E) was purchased from Enzo Life Sciences (www.enzylifesciences.com). All other reagents were purchased from Sigma-Aldrich and Duchefa Biochemie.

Measurement of autophagic flux in cultured cells

The mouse myoblast cell line C2C12 (American Type Culture Collection) was maintained at 37 °C in 5% CO2 in low-glucose DMEM (Gibco, 11885-084) supplemented with 10% fetal bovine serum (Life Technologies, 16010-159), 50 μg/mL penicillin, and 50 μg/mL streptomycin. Cultures were induced to differentiate in DMEM containing 2% horse serum (Life technologies, 16050-122) for 6 days. To measure autophagic flux, C2C12 myotubes grown on 10-cm dishes were incubated in amino acid and serum-free starvation buffer (HBSS, Gibco) or treated with 10 μg/mL rapamycin or 25 μg/mL CCCP with and without 200 nM BafilomycinA1 for 8 h.

Measurement of autophagy flux in animals

Forty-eight 10-week old male wild-type C57BL/6 mice were obtained from the Jackson Laboratory and used in this study. The animals were housed four per cage in a temperature (~22 °C) and light-controlled environment with a 12:12-h light-dark cycle and provided with food and water ad libitum. After 1-week acclimation period, the animals were divided into four groups: fed and starvation, or vehicle and rapamycin. Starvation was performed by removing food for 48 h. Mice had free access to drinking water. Rapamycin was dissolved in 100% ethanol for a 62.5 mg/ml stock solution and stored at 20 °C. Immediately prior to injection, rapamycin was diluted to 2.5 mg/ml in vehicle solution (5% PEG 400, 5% Tween 80 and 4% ethanol). 10 mg/kg/day rapamycin or vehicle was i.p. injected to mice daily for two days as previously described [8]. Colchicine was dissolved in water and stored at −20 °C as a stock solution at a concentration of 4 mg/ml and diluted to 0.1 mg/ml in water prior to injection. 0.4 mg/kg/day colchicine was dosed i.p. daily for two days. Control mice received an equal volume of i.p. saline. Animals were anesthetized using xylazine and ketamine (0.2 ml/g body weight). Tibialis anterior muscles were harvested 24 h after the second dose of rapamycin, CCCP, and colchicine. All protocols for animals use and euthanasia were approved by the University of Suwon Animal Research Ethics Board.

Membrane/cytosol fractionation

All steps were performed on ice using pre-chilled solutions. Centrifugation and incubation were carried out at 4 °C. The fractionations were performed at least three times. Subcellular fractionations from cultured cells or tissue were performed as previously described [9].

Fractionation for cultured cells

C2C12 cells grown on 10-cm dish were washed with room temperature PBS solution and trypsinized by adding 800 μL of 0.25% Trypsin. The cells were incubated at 37 °C for 2 min or until cells are detached. Then 5 mL of culture medium was added to inhibit trypsin activity and cells were scraped into a 15 mL Falcon tube. Scraped cells were pelleted by centrifugation at 500×*g* for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 500 μL of ice cold PBS by pipetting up and down. The cells were centrifuged at 500×*g* for 10 min at 4 °C. The supernatant was discarded and 400 μL of ice cold lysis buffer A (150 mM NaCl, 50 mM HEPES (pH 7.4), 25 μg/mL Digitonin, 1 M Hexylene glycol) supplemented with 4 μL of protease inhibitor cocktail (Sigma, P8340) was added. The pellet was incubated on end-over-end rotator for 10 min at 4 °C. The lysates were centrifuged at 2000×*g* for 10 min at 4 °C. The supernatant were collected and this fraction contains the cytosolic proteins. 200 μL of ice cold lysis buffer B (150 mM NaCl, 50 mM HEPES (pH 7.4), 1% NP-40, 1 M Hexylene glycol) containing protease inhibitors was added to the tube and the pellet was resuspended by vortexing and incubated on ice for 30 min. The suspension was centrifuge at 7000×*g* for 10 min at 4 °C. This supernatant fraction contains the membrane-bound organelle proteins. The protein concentration of cytosolic and membrane fractions was measured using a BCA protein assay kit (Thermo Fisher Scientific). These fractions were stored at −80 °C until analyses were performed.