Published in final edited form as:

Methods Mol Biol. 2018 ; 1801: 111–122. doi:10.1007/978-1-4939-7902-8_11.

Pharmacological Inhibition of Lysosomal Activity as a Method For Monitoring Thyroid Hormone-induced Autophagic Flux in Mammalian Cells In Vitro

Jin Zhou^{#1}, Rohit A. Sinha^{#1,2}, Ronny Lesmana³, Winifred W. Y. Yau¹, and Paul M. Yen^{4,5,6}

¹Program of Cardiovascular and Metabolic Disorders, Duke-NUS Medical School Singapore, Singapore, Singapore

²Department of Endocrinology, SGPGIMS, Lucknow, India

³Department of Physiology, Universitas Padjadjaran, Bandung, Indonesia

⁴Program of Cardiovascular and Metabolic Disorders, Duke-NUS Medical School Singapore, Singapore, Singapore

⁵Duke Molecular Physiology Institute, Department of Medicine, Duke University Medical Center, Durham, NC, USA

⁶Duke Molecular Physiology Institute, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, USA

[#] These authors contributed equally to this work.

Abstract

Autophagy is an evolutionarily conserved intracellular catabolic process that is essential for cellular housekeeping and nutrient homeostasis. Recently, we provided evidence that thyroid hormone (TH) is a major inducer of autophagy in mammalian cells. Here, we describe a method for detecting TH-induced autophagic flux in hepatic, muscle, and brown adipocyte cells using lysosomal inhibitor bafilomycin A1 (BafA1) and conventional Western blot techniques.

Keywords

Thyroid hormone (TH); Autophagic flux; Bafilomycin A1; HepG2; L6 myoblast; Primary brown preadipocytes; LC3B-II

1 Introduction

Macroautophagy (hereafter referred to as autophagy) was first discovered by De Duve in the late 1960s as a glucagon-induced cellular catabolic process in rat liver [1]. Autophagy involves lysosomal-mediated degradation of cellular fuel reserves to provide energy and amino acids during starvation [2]. It is a highly dynamic, multi-step process involving biogenesis of a double membrane structure known as autophagosome, incorporation of

cellular cargoes into the autophagosome, and finally fusion of autophagosome with lysosomes forming autolysosomes for degradation of cellular cargo [3]. The molecular mechanisms of autophagy began to be elucidated shortly after the identification of the various genes involved in this process by the pioneering work of the Ohsumi laboratory [4].

Thyroid hormone signaling has an intricate cross-talk with the autophagy pathway to co-regulate metabolic homeostasis [5]. Indeed, TH is required for several metabolic processes such as lipolysis, fat oxidation, gluconeogenesis, cellular differentiation, mitochondrial biogenesis and thermogenesis [6]. We have recently shown that TH stimulated autophagy in hepatic and muscle cells both in vitro and in vivo. Autophagy-mediated lipolysis of fat droplets termed “lipophagy,” is crucial for hepatic lipolysis and fatty acid oxidation by TH [7, 8]. TH-mediated autophagy also is important for selective mitochondrial clearance via “mitophagy” [9], and protein aggregate removal via “aggrephagy” [10]. Similarly, TH induction of autophagy is necessary for both mitochondrial activity and biogenesis in muscle cells [11].

The detection of active autophagy flux is complex and requires validation by complementary molecular techniques. Autophagic flux refers to the entire process of completed autophagy including the delivery of cargo to lysosomes via autophagosomes, subsequent breakdown of cargo components, and release of the resulting macromolecules back into the cytosol. Although classical methods such as electron microscopy can demonstrate the presence of autophagosomes, they only capture a single time point during autophagy and thus cannot serve as a dynamic readout of autophagic flux. Thus, several methods employing different protein markers of autophagy have been developed to analyze autophagic flux [12].

One of the most commonly used markers for detecting autophagy is the visualization of the protein levels of LC3B-II in cells by Western blotting or immunofluorescence. LC3B is initially synthesized in an unprocessed form pro-LC3B, which is converted to LC3B-I by ATG4B. LC3B-I is further modified into the PE-conjugated form LC3B-II upon induction of autophagy (Fig. 1) [13]. The presence of LC3B-II suggests the formation of autophagosomes, and its level correlates with autophagosomal number. However, an increase in LC3B-II alone cannot serve as a reliable marker of the autophagic flux in cells since it is degraded during the latter stages of autophagy (Fig. 1). Thus, it is possible that a lysosomal block also could lead to an increase in LC3B-II. Similarly, SQSTM1/p62 protein abundance is another commonly used marker to measure autophagic flux. p62 serves as a link between LC3B-II and ubiquitinated substrates which become incorporated into the completed autophagosome and are degraded in autolysosomes, so it can serve as a readout of autophagic degradation[14]. However, several transcription factors may increase the transcription of p62 upon autophagy induction, so an increase in synthesis of p62 may complicate the interpretation of p62 protein levels when used alone as a readout of autophagic flux. Thus, a concomitant increase in LC3B-II and a decrease in SQSTM1/p62 suggest increased autophagic flux. However, there are situations such as rapid autophagic flux when both are unchanged or even decreased.

In this chapter, we will describe a widely accepted technique for assessing autophagic flux that relies on measuring the abundance of LC3B-II protein levels in the absence or presence

of a lysosomal inhibitor bafilomycin A1 (BafA1) using Western blotting. This method relies on the pharmacological inhibition of either lysosomal-autophagosomal fusion or lysosomal acidification by BafA1 [15]. Besides BafA1, there are a variety of pharmacological agents that can be used to block these processes such as chloroquine, lysosomal protease inhibitors, and NH_4Cl (Fig. 1). Using a lysosomal inhibitor such as BafA1 in both TH-treated and non-treated control cells helps to determine whether TH treatment causes an increase in autophagosome formation or a decrease in autophagic degradation.

2 Materials

2.1 Cell Culture Medium and Reagents

1. DMEM, high glucose, HEPES, no phenol red.
2. Fetal bovine serum (FBS).
3. Horse serum.
4. New born calf serum.
5. 0.3% Dowex 1-X10 ion exchange resin.
6. Dowex stripped horse serum.
7. Dowex stripped FBS.
8. Penicillin-Streptomycin (p/s) Solution (100×).
9. DMSO.
10. BafA1.
11. Collagenase.
12. Gentamycin.
13. Isobutylmethylxanthine (IBMX).
14. Dexamethasone.
15. Insulin.
16. Rosiglitazone.
17. Triiodothyronine (T_3).
18. Sodium bicarbonate.
19. Culture flasks and plates.

2.2 SDS-PAGE and Transfer

1. Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8. Weigh 90.85 g Tris-HCl to cylinder. Add water to a volume of 400 ml. Mix and adjust pH to pH 8.8 with HCl. Make up to 500 ml with water. Store at room temperature.
2. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8. Weigh 30.3 g Tris-HCl and prepare a 500 ml solution (pH 6.8) as in previous step. Store at room temperature.

3. 30% Acrylamide/Bis Solution, 29:1.
4. Ammonium persulfate: 10 % solution in water.
5. *N,N,N,N*-tetramethylethylenediamine (TEMED).
6. 10× Tris/Glycine/SDS running buffer: 10× premixed electrophoresis buffer, contains 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 following dilution to 1× with water.
7. Transfer buffer: 192 mM glycine, 25 mM Tris, and 10% methanol.
8. 2× Laemmli Sample Buffer (BIO-RAD 1610737).
9. Dithiothreitol (DTT).
10. PVDF transfer membrane.
11. Molecular weight marker.
12. Ponceau S solution.

2.3 Immunoblotting

1. Nonfat milk powder.
2. PBS containing 0.1% Tween 20 (PBST).
3. Blocking buffer: 5% milk in PBST.
4. Primary antibody dilution buffer: 1% BSA in PBST.
5. Secondary antibody dilution buffer: 5% milk in PBST.
6. Anti-LC3B antibody (Cell Signaling, Cat #2775).
7. Anti- β -actin antibody.
8. Mouse anti-rabbit IgG-HRP.
9. Chicken anti-mouse IgG-HRP.
10. Enhanced chemiluminescent substrate ECLTM Select Western Blotting Detection Reagent (GE Healthcare, RPN2235).

3 Methods

Determination of Autophagic Flux Using BafA1 in vitro

We have used the following protocols to study autophagic flux in HepG2-TR β , L6 myoblasts, and primary brown preadipocytes, respectively. We perform in vitro BafA1 treatments to assess autophagic flux in each cell line of interest. We have found that the efficiency of BafA1-induced lysosomal inhibition varies in different cell types. Therefore, we highly recommend performing a dose response/time course of BafA1 in the cell line of interest in order to identify the optimal dose and duration of BafA1 treatment.

3.1 Cell Culture and BafA1 Treatment

3.1.1 HepG2 Cells Over-Expressing THR α or THR β

Culture of HepG2 Cells Over-Expressing THR α or THR β

1. Maintain HepG2 cells with stably transfected human *THR α /B* at 37 °C in DMEM supplemented with 10% FBS as described previously [8].
2. Plate cells in 6- or 12-well plates followed by culture in TH-depleted media (DMEM + 10% Dowex-stripped FBS) [8], for 2 days before adding T₃ (100 nM) for 48 h.
3. For autophagic flux analysis, add 100 nM BafA1 to the culture medium 6 h before protein isolation.

3.1.2 L6 Myoblasts

Culture of L6 Myoblasts: L6 cells are commercially available from American Type Culture Collection (ATCC, Manassas, VA, USA). Please note that variable handling of this cell line can select for cells with different growth kinetics or poor differentiation. To avoid these problems, please make sure that low passage L6 Stocks are used initially and low-passage cells (<passage 10) for each experiment in order to maintain original cell characteristics.

Maintain L6 cells in L6 proliferation medium (DMEM, 15% FBS, p/s, and 1mM sodium bicarbonate). The cells are grown on Nunc delta surface plastic culture flasks. Cells are cultured at 37 °C in 20 ml of proliferation medium in a humidified incubator with 5% CO₂ for 48 h. Cell density is maintained at approximately 70% density (typically found 72 h after initial plating at 10% density in 75 cm² flasks).

1. Remove and discard culture medium.
2. Briefly rinse the cells with Phosphate-Buffered Saline (PBS) $\times$ 2 times.
3. Add 2.0 ml of Trypsin EDTA, swirl to cover all surface cells and incubate for 2 min in 37 °C.
4. Check under a microscope whether cells are detached from the flask surface.
5. Resuspend cells with 5 ml fresh warm medium and centrifuge them for 3 min with 1300 $\times$ g.
6. Discard the supernatant and resuspend pellets using 5 ml medium.
7. Add appropriate aliquots of the cell suspension to new culture vessels.
8. Incubate cultures at 37 °C.

Differentiation and Treatment of L6 Cells

1. Grow cells to 40–50% density in 12- or 24-well plates with proliferation medium.
2. Check growth of cells after 24 h (make sure that cells are attached to the bottom of plate).

3. Change medium to L6 differentiation medium (DMEM, 2% Horse serum, p/s, and 1 mM sodium bicarbonate). Cells typically reach 90–100% confluency after 48 h.
4. Incubate the cells in differentiation medium for 4–7 days.
5. Change medium for every 48 h.
6. Replace L6 differential medium with L6 treatment medium (DMEM, 2% Dowex stripped horse serum, p/s, and 1 mM sodium bicarbonate) after cells are fully differentiated, and culture the cells for 2 days. Cells are then treated in the presence or absence of 10 nM T₃ for 24–48 h [11]. For autophagic flux analysis, L6 cells are co-treated with 30 nM of BafA1 for 24 h in the presence or absence of 100 nM T₃ for 24 h.

3.1.3 Primary Brown Preadipocytes

Primary Culture of Brown Preadipocytes

1. Harvest interscapular brown adipose tissue from five to six mice (2–3 weeks old).
2. Mince tissue using scissors in a 10 cm plate with collagenase (300 µl).
3. Transfer the tissue to a 50 ml tube and add another 2.7 ml of collagenase.
4. Put the tube on a thermal shaker at 37 °C for 30–40 min. Vortex for 10 s every 5 min.
5. Add 10 ml plain DMEM to the digestion mixture and filter through a 100 µm mesh into a 15 ml tube.
6. Centrifuge at $330 \times g$ at 4 °C for 5 min. Remove the supernatant.
7. Resuspend the pellet with 10 ml of plain DMEM and filter through a 100 µm mesh.
8. Centrifuge at $330 \times g$ at 4 °C for 5 min. Remove the supernatant.
9. Resuspend the pellet with 10 ml of culture medium (DMEM containing 10% new born calf serum, gentamycin and p/s) and transfer to a new 10 cm culture plate.

Differentiation and Treatment of Brown Adipocytes

1. Seed brown preadipocytes onto 12-well plates for differentiation.
2. Initiate adipogenesis on Day 0 with induction medium (DMEM containing 10% FBS, 0.5 mM IBMX, 1 µM dexamethasone, 172 nM insulin, 1 nM T₃ and 1 µM rosiglitazone).
3. Change medium to culture medium supplemented with 172 nM insulin on Day 2.
4. Change medium to T₃-depleted culture medium on Day 4.

5. On Day 6, mature brown adipocytes are ready for experimental treatment. Incubate cells with T₃-depleted culture medium containing 10 nM T₃ for 24 h. For autophagic flux analysis, add 50 nM BafA1 to the medium 6 h before harvest.

3.2 LC3B SDS-PAGE and Western Blot Analysis

3.2.1 Preparation of Protein Samples from Cultured Cells—Make 1× cell lysis buffer by diluting 2× Laemmli sample buffer with H₂O and 1 M DTT. 100 µl/well is then added to 12-well plates. Of note, T₃ treatment usually does not affect cell number under our experimental conditions. If the cell number changes during the experiment, we recommend lysing the cells with RIPA buffer followed by measurement of protein concentrations, and adjusting the protein concentration to the same levels before mixing the protein samples with 2× Laemmli sample buffer.

1. Prepare 1× cell lysis buffer by adding 2× Laemmli sample buffer, H₂O, 1 M DTT in a volume distribution of 5:4:1.
2. Rinse the cells with PBS. Lyse the cells by adding 100 µl 1× cell lysis buffer. Stir the lysis buffer using pipet tips until the lysis buffer becomes very viscous. Try to transfer the entire contents into 1.5 ml Eppendorf tube.
3. Boil the samples at 100 °C for 10 min. Spin down the samples for 30 s. Cool down the samples to room temperature. Protein samples are now ready for SDS-PAGE or can be stored up to one month at −30 °C.

3.2.2 LC3B SDS-PAGE and Immunoblot Analysis—14% resolving gel is recommended for LC3B SDS-PAGE since it provides good separation of the LC3B-I and LC3B-II bands.

1. Load 10 µl of protein sample using gel loading tip. Run PAGE using 1× SDS running buffer.
2. Soak the PVDF membrane with 100% methanol for approximately 1 min while the gel is running. The methanol can be reused again for this same purpose. Rinse the membrane with distilled water. Soak the membrane in 1× transfer buffer (Transfer buffer: 192 mM glycine, 25 mM Tris, and 10% methanol) in a separate container. Keep the PVDF membranes and filter papers refrigerated.
3. Stop the electrophoresis when the dye front reaches the very bottom of the gel.
4. Transfer onto a PVDF membrane using constant voltage at 25 V for 30 min at room temperature using Trans-Blot® Turbo™ Blotting System (Bio-Rad).
5. Stain the membrane with Ponceau S solution to visualize the transfer efficiency. Ponceau S binding to protein is reversible and can later be washed away in blocking buffer.
6. Rinse the membrane with distilled water to remove excess Ponceau S solution.
7. Block in blocking buffer for 1 h at room temperature on a shaker.

8. Rinse the membrane with 1× PBST.
9. Prepare the rabbit-anti-LC3B and mouse-anti-actin primary antibodies by diluting them 1:1000 in 5 ml of 1% BSA in PBST in a 50 ml Falcon tube.
10. Incubate the membrane with primary antibodies on Roller Mixer (Ratek Laboratory) inside the cold room.
11. Wash the membrane with PBST for 10 min at room temperature ×3 on the shaker.
12. Prepare secondary antibody by diluting it 1:5000 in 5% milk in PBST. Incubate the membrane in secondary antibody solution for 1 h at room temperature on the shaker.
13. Wash the membrane with PBST for 10 min at room temperature ×3 on the shaker.
14. Develop the blots with Enhanced Chemiluminescent Substrate ECL™ Select Blotting Detection Reagent using Gel Doc™ XR and Gel Documentation System (BIO-RAD).
15. Quantify LC3B-II, and actin protein bands using ImageJ and report the data as LC3B-II/actin.

4 Interpretation of Results

The level of cellular LC3B-II at any given point in time is a snapshot based upon the rate of autophagosome biogenesis and degradation after lysosomal fusion. Therefore, the use of lysosomal inhibitors to analyze autophagic flux enables one to ascertain the rate at which autophagosome formation is being induced by a drug/hormone or nutritional state when its degradation is stopped. In other words, any increase in the levels of LC3B-II that occurs in the presence of a lysosomal inhibitor is due to autophagy induction or autophagosome biogenesis prior to, and during, the period when lysosomal activity was blocked. The following are some potential scenarios. In Fig. 2 scenario 1: the relative increase in LC3B-II/ACTB in treated cells plus BafA1 compared to treated cells alone is greater than the increase in LC3B-II/ACTB in control cells (untreated) plus BafA1 compared to control cells alone, suggests that there is an increase in autophagic flux. An increase in LC3B-II levels after lysosomal activity is inhibited by BafA1 can only be explained by increased autophagy induction. In cases, where LC3B-II levels are unchanged or even decreased, an increase in LC3B-II level after BafA1 treatment can be due to high autophagic flux, particularly if p62 levels are decreased without Baf1 treatment. On the contrary, in Fig. 2 scenario 2: the relative increase in LC3B-II/ACTB in treated cells plus BafA1 compared to treated cells alone is less than the increase in LC3B-II/ACTB in control cells + BafA1 compared to control cells alone, it is likely that autophagosome accumulation (LC3B-II) observed after treatment is due to inhibition of autophagic degradation rather than increased autophagosome synthesis. In this case, blocking autophagosome-lysosome fusion or lysosomal function decreases net autophagic flux. A real example of scenario 1 is shown in Fig. 3, where T₃ increases autophagic flux as described in Fig. 1.

When interpreting initial autophagy results, it is critical to ascertain whether an increase in LC3B-II is due to increased biosynthesis or degradation of autophagosomes, and reflects a genuine increase in autophagic flux. While an increase in LC3B-II with a concomitant decrease in p62 levels is highly suggestive of increased autophagy, formal confirmation using a lysosomal inhibitor such as BafA1 is necessary to prove autophagic flux. In general, it is highly recommended to monitor autophagy flux by using at least two techniques. Detection of autophagic flux by transfecting tandem mRFP-GFP-LC3 vector followed by fluorescent imaging-based analysis of yellow and red fluorescence in live or fixed cells are widely used techniques [12, 16]. Another probe to detect autophagic flux both in vitro and in vivo has been developed recently. This GFP-LC3-RFP-LC3 G probe can be cleaved by endogenous ATG4 protease into equimolar amounts of GFP-LC3 and RFP-LC3 G. GFP-LC3 is degraded by autophagic flux, whereas RFP-LC3 G remains in the cytosol, serving as an internal control. Thus, autophagic flux can be estimated by calculating the GFP/RFP ratio, which is inversely correlated with autophagic flux [17]. Additionally, increases in lysosomal activity can be monitored by using acridine orange [18] or monodansylcadaverine staining [19].

Acknowledgement

This work was supported by Duke-NUS Medical School Faculty Funds (to P.M.Y.), Singapore National Medical Research Council grant NMRC/CSA/0054 /2013 (to P.M.Y.), NMRC/CIRG/1340/2012 (to P.M.Y.), NMRC/CIRG/1457/2016 (to P.M.Y.), Wellcome Trust/DBT India Alliance Fellowship (IA/I/16/2/502691) to R.A.S.

References

1. Deter RL, Baudhuin P, De Duve C. Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. *J Cell Biol.* 1967; 35:C11–C16. [PubMed: 6055998]
2. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol.* 2013; 15:713–720. [PubMed: 23817233]
3. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res.* 2014; 24:24–41. [PubMed: 24366339]
4. Tsukada M, Ohsumi Y. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* 1993; 333:169–174. [PubMed: 8224160]
5. Sinha RA, Singh BK, Yen PM. Reciprocal crosstalk between autophagic and endocrine signaling in metabolic homeostasis. *Endocr Rev.* 2016; 2016:er20161103.
6. Brent GA. Mechanisms of thyroid hormone action. *J Clin Invest.* 2012; 122:3035–3043. [PubMed: 22945636]
7. Tseng YH, Ke PY, Liao CJ, Wu SM, Chi HC, Tsai CY, Chen CY, Lin YH, Lin KH. Chromosome 19 open reading frame 80 is upregulated by thyroid hormone and modulates autophagy and lipid metabolism. *Autophagy.* 2014; 10:20–31. [PubMed: 24262987]
8. Sinha RA, You SH, Zhou J, Siddique MM, Bay BH, Zhu X, Privalsky ML, Cheng SY, Stevens RD, Summers SA, Newgard CB, et al. Thyroid hormone stimulates hepatic lipid catabolism via activation of autophagy. *J Clin Invest.* 2012; 122:2428–2438. [PubMed: 22684107]
9. Sinha RA, Singh BK, Zhou J, Wu Y, Farah BL, Ohba K, Lesmana R, Gooding J, Bay BH, Yen PM. Thyroid hormone induction of mitochondrial activity is coupled to mitophagy via ROS-AMPK-ULK1 signaling. *Autophagy.* 2015; 11:1341–1357. [PubMed: 26103054]
10. Chi HC, Chen SL, Tsai CY, Chuang WY, Huang YH, Tsai MM, Wu SM, Sun CP, Yeh CT, Lin KH. Thyroid hormone suppresses hepatocarcinogenesis via DAPK2 and SQSTM1-dependent selective autophagy. *Autophagy.* 2016; 12:2271–2285. [PubMed: 27653365]

11. Lesmana R, Sinha RA, Singh BK, Zhou J, Ohba K, Wu Y, Yau WW, Bay BH, Yen PM. Thyroid hormone stimulation of autophagy is essential for mitochondrial biogenesis and activity in skeletal muscle. *Endocrinology*. 2016; 157:23–38. [PubMed: 26562261]
12. Klionsky DJ, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. 2016; 12:1–222. [PubMed: 26799652]
13. Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. *J Biol Chem*. 2003; 278:51841–51850. [PubMed: 14530254]
14. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*. 2005; 171:603–614. [PubMed: 16286508]
15. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. *Autophagy*. 2007; 3:542–545. [PubMed: 17611390]
16. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy*. 2007; 3:452–460. [PubMed: 17534139]
17. Kaizuka T, Morishita H, Hama Y, Tsukamoto S, Matsui T, Toyota Y, Kodama A, Ishihara T, Mizushima T, Mizushima N. An autophagic flux probe that releases an internal control. *Mol Cell*. 2016; 64:835–849. [PubMed: 27818143]
18. Paglin S, Hollister T, Delohery T, Hackett N, McMahon M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res*. 2001; 61:439–444. [PubMed: 11212227]
19. Munafo DB, Colombo MI. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J Cell Sci*. 2001; 114:3619–3629. [PubMed: 11707514]

Autophagic induction

Fig. 1.

The turnover of LC3B-II. This schematic sketch shows the dynamics of LC3B-II during the process of autophagy. Upon autophagy induction due to either energy or nutrient depletion, the protease ATG4B cleaves Pro-LC3B to form mature LC3B-I. Concomitantly, LC3B-I that is cytosolic is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3B-II) via other ATG family proteins, which is recruited to autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components including LC3B-II are degraded by lysosomal hydrolases. Therefore, the levels of LC3B-II detected inside the cells are a reflection of the net balance between the rate at which the autophagosomes are being made and the rate at which they are being degraded during autolysosome formation. Use of BafA1 inhibits both the autophagosome-lysosome fusion as well as lysosomal acidification serves to block the completion of the autophagic flux in cells

Fig. 2. Interpretation of the LC3B-II levels as a readout of autophagic flux. This figure represents a Western blot result showing the levels of LC3B-I, LC3-II and β -Actin (ACTB) upon probing with anti-LC3B and anti-ACTB antibodies

Fig. 3.

T₃ increases autophagic flux in vitro. A representative Western blot and densitometric analysis of LC3B-II/ACTB ratio showing increased autophagic flux in T₃ treated HepG2 overexpressing THR β