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Cholesterol depletion induces autophagy

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Abstract

Autophagy is a mechanism to digest cells' own components, and its importance in many physiological and pathological processes is being recognized. But the molecular mechanism that regulates autophagy is not understood in detail. In the present study, we found that cholesterol depletion induces macroautophagy. The cellular cholesterol in human fibroblasts was depleted either acutely using 5 mM methyl-β-cyclodextrin or 10–20 μg/ml nystatin for 1 h, or metabolically by 20 μM mevastatin and 200 μM mevalonolactone along with 10% lipoprotein-deficient serum for 2–3 days. By any of these protocols, marked increase of LC3-II was detected by immunoblotting and by immunofluorescence microscopy, and the increase was more extensive than that caused by amino acid starvation, i.e., incubation in Hanks' solution for several hours. The induction of autophagic vacuoles by cholesterol depletion was also observed in other cell types, and the LC3-positive membranes were often seen as long tubules, >50 μm in length. The increase of LC3-II by methyl-β-cyclodextrin was suppressed by phosphatidylinositol 3-kinase inhibitors and was accompanied by dephosphorylation of mammalian target of rapamycin. By electron microscopy, autophagic vacuoles induced by cholesterol depletion were indistinguishable from those seen after amino acid starvation. These results demonstrate that a decrease in cholesterol activates autophagy by a phosphatidylinositol 3-kinase-dependent mechanism.

Keywords: Autophagy; Cholesterol; Phosphatidylinositol 3-kinase; LC3; Mevastatin

Autophagy is a process of bulk degradation, which exists from yeast to mammals [1,2]. Macroautophagy, a dominant form of autophagy in mammalian cells, is important for the turnover of long-lived proteins and as a survival mechanism during starvation. Moreover, recent studies revealed the engagement of autophagy in the disposal of toxic protein aggregates [3,4] and defense against bacterial infections [5]. On the other hand, excessive activation of autophagy can lead to a type of cell death that is distinct from apoptosis [6]. Involvement of autophagy in some aspects of cancer progression has been also shown [7]. These findings suggest that the level of autophagy needs to be regulated within a certain range and that either excessive or deficient autophagy may cause various diseases.

In mammalian cells, macroautophagy can be triggered by nutrient starvation, stress, or treatment with some hormones. These signals lead to the dephosphorylation and inactivation of mammalian target of rapamycin (mTOR), which then induces autophagy [8]. Rapamycin activates autophagy by inhibiting mTOR, whereas lithium induces autophagy by a mechanism independent of mTOR [9]. Activation of class III phosphatidylinositol-3-kinase (PI3K) is involved in the autophagosome formation, and PI3K inhibitors such as 3-methyladenine (3-MA) inhibit autophagy [10]. But how the inactivation of mTor and activation of class III PI3K are correlated is not known. Our understanding of the regulatory mechanism of autophagy is far from complete.

In the present study, we found serendipitously that depletion of cholesterol triggers autophagy. Cholesterol depletion has been used extensively to study the role of cholesterol in many biological processes, especially in relation to membrane microdomains [11,12], but induction of

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autophagy has not been described. Our result indicates that autophagy is one outcome of cholesterol depletion and needs to be considered when interpreting long-term effects of this manipulation. More importantly, these results revealed that perturbation of the cellular lipid environment could induce autophagy and suggests a possibility that pharmacological reagents that influence the lipid metabolism may be used to modulate the level of autophagy in vivo.

Materials and methods

Cells and antibodies. Human and mouse fibroblasts and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS). Stable Huh7 cell lines expressing GFP-LC3 were generated by using a vector kindly provided by Dr. Tamotsu Yoshimori [13]. Rabbit anti-LC3 antibody was donated by Dr. Yasuo Uchiyama. Rat and mouse anti-LAMP1 antibodies (Developmental Studies Hybridoma Bank at the University of Iowa), rabbit anti-mTOR and rabbit anti-phospho-mTOR antibodies (Cell Signaling), and fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Lab) were purchased.

Cholesterol depletion. For acute cholesterol depletion, cells were treated for 60 min with either 5 mM methyl-β-cyclodextrin (MeβCD; Sigma) or 10–20 μg/ml nystatin (Sigma) in DME. For metabolic cholesterol depletion, cells were cultured in 10% lipoprotein-deficient serum (LPDS) in the presence of 20 μM mevastatin and 200 μM mevalonolactone (Sigma). For amino acid starvation, cells were incubated in Hepes-buffered Hanks' solution. Five millimolar MeβCD–cholesterol complex in DME was used for cholesterol supplementation.

Western blotting. Cell lysates containing an equal protein amount (20–30 μg) were electrophoresed in acrylamide gels and subjected to Western blotting. After incubation with HRP-conjugated secondary antibodies (Pierce), the blots were developed using SuperSignal West Dura Substrate (Pierce).

Microscopy. Immunofluorescence labeling was done as described [14]. Images were captured with an AxioCam CCD camera (Carl Zeiss) and the contrast of the images was adjusted using Adobe Photoshop 7.0. For electron microscopy, cells were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, stained with uranyl acetate, and embedded in Epon. Ultrathin sections were observed using a JEOL 1200EX electron microscope.

Results and discussion

To induce autophagy by amino acid starvation, we cultured human fibroblasts in Hanks' solution and monitored the relative ratio of LC3-II/LC-I by Western blot. LC3 is a mammalian homolog of Atg8 [15] and exists mostly as a soluble cytosolic protein, or LC3-I, at the normal culture condition. Upon induction of autophagy, LC3-I is modified with phosphatidylethanolamine, and incorporated into the autophagic membrane as LC3-II. By Western blotting, LC3-I and LC3-II are seen as bands at 18 kDa and 16 kDa, respectively. Incubation in Hanks' solution for 2 h induced a slight increase of LC3-II in human fibroblasts, whereas cholesterol depletion caused a much stronger induction of LC3-II within 1 h (Fig. 1A). To reduce the membrane cholesterol acutely, cells were incubated for 1 h with 5 mM MeβCD in DME or with 10–20 μg/ml nystatin in DME. MeβCD extracts free cholesterol from membranes with a high specificity [16], whereas nystatin makes a complex with membrane cholesterol [17]. The two reagents gave comparable results with regard to the increase of the LC3-II/LC3-I ratio. The increase of LC3-II was not observed when cells were incubated in DME without additional reagents for 1 h (Fig. 1A). This result indicated that the lack of serum in DME did not induce autophagy due to nutrient insufficiency and that depletion of cholesterol was the main cause of the LC3-II increase. Likewise, lack of cholesterol in the medium did not contribute to the autophagic induction by Hanks' solution because addition of 5 mM MeβCD–cholesterol complex did not suppress the increase of LC3-II (data not shown).

We next examined the time course of LC3-II induction by MeβCD. The LC3-II band became apparent as early as 30 min after the addition of MeβCD, and its intensity increased as the incubation was extended longer (Fig. 1A). Treatment with MeβCD has been used extensively to study the effect of cholesterol depletion on various cellular phenomena, particularly in relation to membrane microdomains [11,12]. The incubation with 5 mM MeβCD for 30–60 min is an average protocol for such experiments. In many such experiments, to show the reversibility of the reaction, cholesterol was added to the medium as a complex with MeβCD after the initial depletion. However, with regard to autophagy, the increase of LC3-II induced by cholesterol depletion persisted for several hours, even after cholesterol was replenished (data not shown). This result suggests that occurrence of autophagy may need to be taken into account when interpreting long-term effects of cholesterol depletion on various cellular activities.

MeβCD is effective in reducing the membrane cholesterol acutely, but cholesterol depletion by this reagent has been reported to cause some effects that are not observed when the cellular cholesterol is decreased by milder procedures [18,19]. To examine whether the LC3-II induction also occurs by slower cholesterol reduction, cells were kept in 10% LPDS in the presence of 20 μM mevastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, and 200 μM mevalonolactone. Mevalonolactone was added to replenish mevalonate for isoprenoid synthesis. The induction of LC3-I was not apparent after 1 day, but became significant after 2 days (Fig. 1A). The same result was obtained when lovastatin was used instead of mevastatin (data not shown). Thus the increase of LC3-II was caused even when the cellular cholesterol was reduced metabolically.

The effect of several other procedures that could change the intracellular cholesterol distribution was also examined. Human fibroblasts were subjected to the following conditions for 3 days: 1 μM U18666A, 10% LPDS alone, or 20 μM mevastatin/200 μM mevalonolactone in 10% FCS. U18666A caused a significant increase in LC3-II (Supplementary Fig. 1). U18666A suppresses the export of free cholesterol from late endosomes, mimicking Niemann-Pick type C disease. This result may be explained by a decrease of cholesterol in the cell membrane. On the other hand, LPDS and mevastatin/mevalonolactone

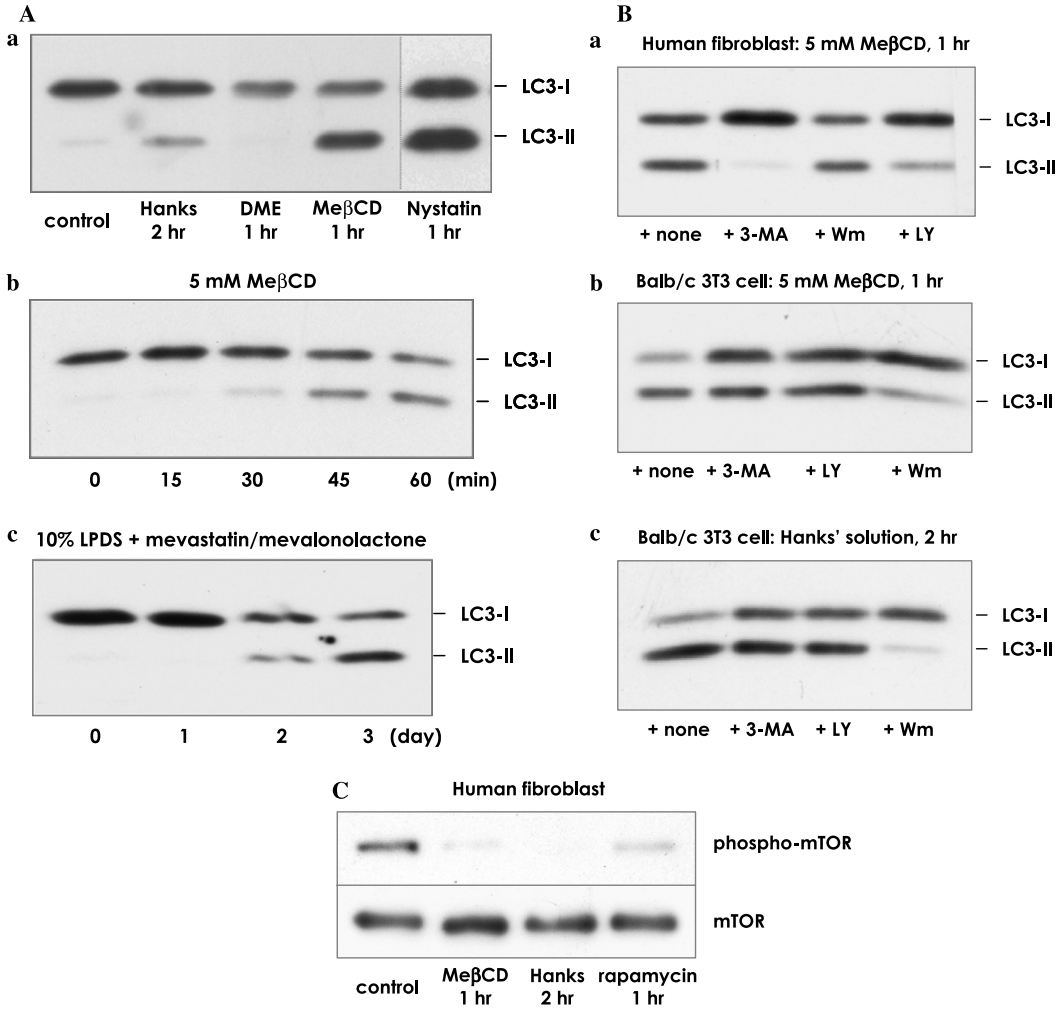

Fig. 1. Western blots. (A) Induction of LC3-II by cholesterol depletion. (a) Total lysates of human fibroblasts were examined for expression of LC3 by Western blotting. Cells cultured in control medium, Hanks' solution for 2 h, DME for 1 h, 5 mM MeβCD in DME for 1 h, 10 μg/ml nystatin in DME for 1 h, and 10% LPDS plus 20 μM mevastatin and 200 μM mevalonolactone for 2 days were used. LC3-II was negligible in the control but increased significantly after cholesterol depletion. Incubation in Hanks' solution was not effective for LC3-II induction in this cell type. (b) The time course of LC3-II induction by MeβCD treatment. The LC3-II band became visible after 30–45 min of treatment. (c) The time course of LC3-II induction by LPDS plus mevastatin/mevalonolactone. The LC3-II band became prominent on day 2 and further intensified on day 3. (B) Effect of PI3K inhibitors on LC3-II induction. (a) The induction of LC3-II in human fibroblasts by MeβCD was blocked significantly by either 3-MA or LY294002 (LY) but was not affected significantly by wortmannin (Wm). (b) The induction of LC3-II in mouse Balb/c 3T3 cells by MeβCD was blocked significantly by wortmannin (Wm) but less effectively by 3-MA or LY294002 (LY). (c) The effect of the PI3K inhibitors on LC3-II induction by amino acid starvation in Balb/c 3T3 cells showed a similar profile to that by cholesterol depletion: wortmannin (Wm) was more effective than 3-MA or LY294002 (LY). (C) Total and phosphorylated mTOR. Human fibroblasts were cultured in normal medium, 5 mM MeβCD for 1 h, Hanks' solution for 2 h, or 5 μM rapamycin for 1 h. The amount of phosphorylated mTOR was reduced drastically by any of these three treatments.

should reduce the uptake and de novo synthesis of cholesterol, respectively, but neither of them increased LC3-II (Supplementary Fig. 1). The result suggests that the total cholesterol content is critical and that whether cholesterol is supplied by exogenous lipoproteins or through endogeneous synthesis does not matter.

PI3Ks are involved in the regulation of autophagy, and PI3K inhibitors have been shown to suppress autophagy induced by amino acid starvation [10]. To study whether PI3K inhibition affects autophagy induced by cholesterol depletion, the effects of three inhibitors were examined: 10 mM 3-MA, 20 μM LY290042, and 100 nM wortmannin. When added to 5 mM MeβCD in DME, the increase of LC3-II in human fibroblasts was suppressed by 3-MA or LY290042, but not by wortmannin (Fig. 1B). On the other hand, in Balb/c 3T3 cells, wortmannin was more effective than 3-MA or LY290042 (Fig. 1B). The difference among the three inhibitors was observed similarly when autophagy was induced by amino acid starvation (Fig. 1B), suggesting that the effectiveness of respective PI3K inhibitors may be varied in different cells.

The above result suggested that PI3K is involved in the induction of autophagy by cholesterol depletion in a similar manner to that by amino acid starvation. To further examine whether the two conditions induced autophagy by a common mechanism, activation of mTOR was studied

by using an anti-phospho-mTOR antibody. The samples treated by acute cholesterol depletion, amino acid starvation, and rapamycin all showed a significant reduction of phospho-mTOR (Fig. 1C), indicating that inactivation of mTOR occurred similarly in all three conditions. This result demonstrated that autophagy upon cholesterol depletion is induced by the same mechanism as amino acid starvation, acting downstream of mTOR inactivation.

Distrubution of LC3-positive autophagic vacuoles was examined by immunofluorescence microscopy (Fig. 2). In human fibroblasts, endogenous LC3 was seldom observed as distinct points when the cells were cultured in the standard medium containing 10% FCS. After incubating in Hanks' solution for 2 h, many cells showed LC3 labeling as small dots (Fig. 2A). Acute cholesterol depletion by 5 mM MeβCD or by 10–20 μg/ml nystatin also induced a similar punctate LC3 labeling pattern. In accordance with the Western blotting, labeling after cholesterol depletion was more prominent than after incubation in Hanks' solution. When cholesterol was depleted by incubation with 10% LPDS and 20 μM mevastatin/200 μM mevalonolactone for more than 2 days, labeled LC3 appeared as distinct dots. The dots were relatively large in those cells, and occasionally labeling in large circular profiles and long curvilinear lines was observed. The curvilinear labeling was seen more clearly in mouse dermal fibroblasts with wide flat cytoplasm (Supplementary Fig. 2).

A very similar result was obtained in Huh7 cells that stably express GFP-LC3 (Fig. 2C). Cells kept in the normal culture medium did not show distinct labeling, but both acute cholesterol depletion by MeβCD and amino acid starvation by the Hanks' solution induced GFP-positive dots in most cells. Metabolic cholesterol depletion by LPDS and mevastatin/mevalonolactone induced larger points of GFP-LC3, which often showed circular profiles. Curvilinear labeling was also observed in these cells.

The increase of LC3-II is likely to represent the increased generation of autophagosomes, but suppression of LC3-II degradation may cause a similar result [15]. The large LC3-positive structures induced by LPDS and mevastatin/mevalonolactone imply the possibility of the latter phenomenon. To explore this, autophagic vacuoles induced by different protocols were observed by electron microscopy (Fig. 3). Autophagosomes containing phagocytosed materials were observed, and their frequency was in accordance with the intensity of LC3 labeling. Morphology of autophagosomes induced by acute cholesterol depletion was not different from that seen upon amino acid starvation. The result suggests that autophagy induced by acute cholesterol depletion proceeded in a manner similar to that of amino acid starvation within the time frame examined, i.e., 1–2 h. On the other hand, multilamellar figures were conspicuous in cells treated by LPDS and mevastatin/mevalonolactone for 2–3 days. The structure probably represents remnants left after lysosomal degradation. Because the duration of the treatment for metabolic cholesterol depletion was much longer than others, direct comparison is not possible. But the observation suggested that most, if not all, autophagosomes fused with late endosome/lysosomes in cells treated by LPDS and mevastatin/mevalonolactone.

If retardation in the autophagic vacuole maturation occurs, LAMP1, a late endosome/lysosome maker, should colocalize extensively with LC3, because LC3 would be

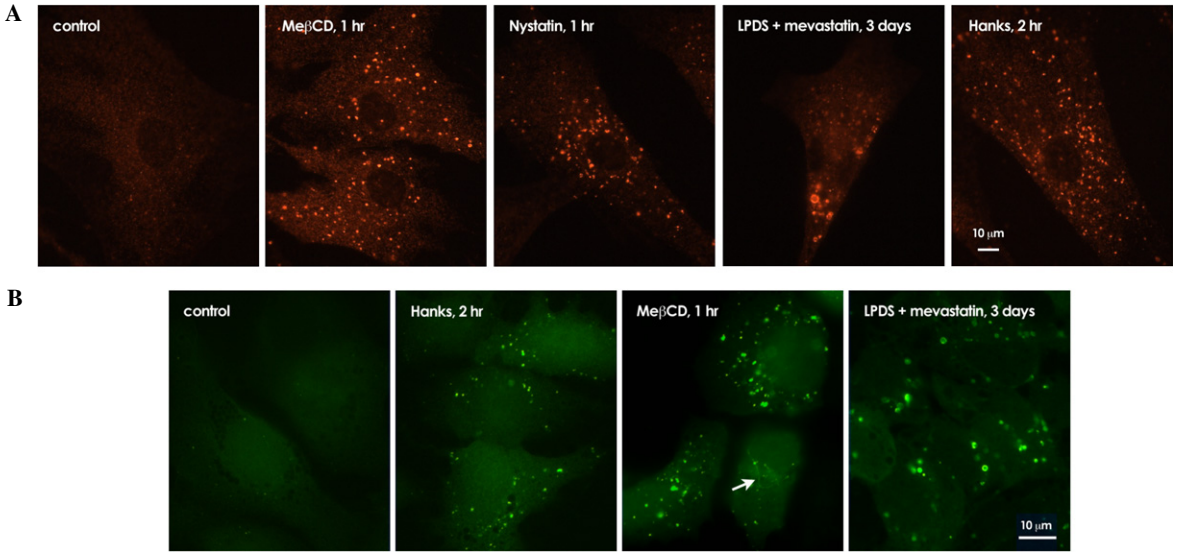

Fig. 2. Immunofluorescence microscopy of LC3. (A) Human fibroblasts. LC3 was seen as diffuse background in cells cultured in the normal medium. After incubation in Hanks' solution for 2 h, LC3 was seen as distinct dots in many cells. The punctate labeling was also prominent when cholesterol was depleted by 5 mM MeβCD or 10 μg/ml nystatin for 1 h. Cells cultured in LPDS and mevastatin/mevalonolactone for 3 days also showed a similar LC3 labeling. (B) Huh7 cells expressing GFP-LC3. Fluorescence in cells cultured in the normal medium was not distinct, but after incubation in Hanks' solution for 2 h, GFP-LC3 was seen as small dots. After depleting cholesterol by MeβCD or LPDS plus mevastatin/mevalonolactone, the GFP-LC3 dots became larger, and some appeared in a donut-shape. Curvilinear labeling was observed in cells cultured in LPDS and mevastatin/mevalonolactone (arrow).

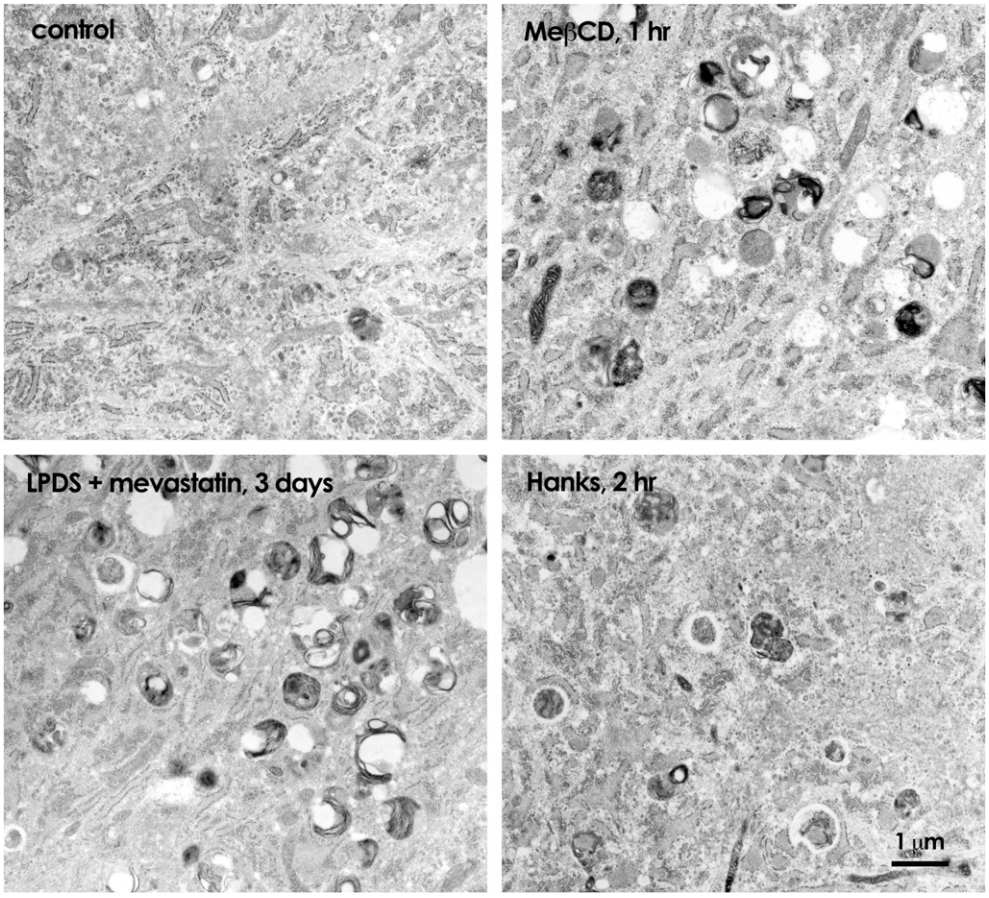

Fig. 3. Electron microscopy of human fibroblasts Cells were cultured in control medium, 5 mM MeβCD for 1 h, 10% LPDS plus mevastatin/mevalonolactone for 3 days, or Hanks' solution for 2 h and subjected to conventional electron microscopy. Autophagosomes seen after amino acid starvation were very similar to those induced by MeβCD, whereas those induced by prolonged cholesterol depletion often contained multilamellar structures, which probably represent a later stage of autophagy.

degraded less effectively than in the normal process. Cells treated by different protocols were double-labeled for LC3 and LAMP1, and compared (Fig. 4A). Colocalization of LC3 and LAMP1 was seen only sporadically in most cells cultured in LPDS and mevastatin/mevalonolactone, and the degree to which the two markers colocalizated was not different from that seen in cells where autophagy was induced by acute cholesterol depletion, amino acid starvation, or rapamycin. The long curvilinear LC3-positive structures were not labeled for LAMP1, either (Supplementary Fig. 2). On the other hand, in the large circular LC3-positive structures seen occasionally in cells cultured in LPDS and mevastatin/mevalonolactone, weak LAMP1 labeling was often observed coincidentally (Fig. 4B). This result implied that upon prolonged cholesterol depletion, some degree of retardation may occur in the maturation process of autophagosomes. In this context, it is noteworthy that filipin cytochemistry did not detect cholesterol in nascent autophagic vacuoles but showed dense labeling in older ones [20]. Cholesterol may not be necessary for autophagosomal formation but may be required in later stages.

The mechanism by which cholesterol depletion initiates autophagy is not clear, but disruption of membrane rafts might be involved. Among several proteins engaged in the autophagic regulation, basal and growth factor-induced Akt activity was shown to be dependent on the raft integrity [21,22]. Thus a simple scenario is that cholesterol depletion disrupts membrane rafts, and the resultant down-regulation of Akt activity leads to suppression of mTOR and induced autophagy. This putative signaling pathway is largely in common with autophagy induced by amino acid starvation. The observation that autophagy in either case was susceptible to PI3K inhibitors is consistent with this speculation.

Considering the possible involvement of autophagy in various diseases, the importance of understanding its regulatory mechanism is obvious. Recent studies revealed that suppression of autophagy in the adult mouse brain causes neurodegeneration [3,4], indicating the critical role of autophagy in disposing toxic protein aggregates in the central nervous system. Pharmacological reagents that modulate the level of autophagy can be potential therapeutic tools, but there are not many candidates at present. In this context, reduction of cholesterol in vivo could be a realistic measure because statins have been used clinically for many years. However, for Alzheimer's disease, it could be a double-edged sword: on one hand, a decrease of cholesterol should inhibit generation of β-amyloid [23,24] and

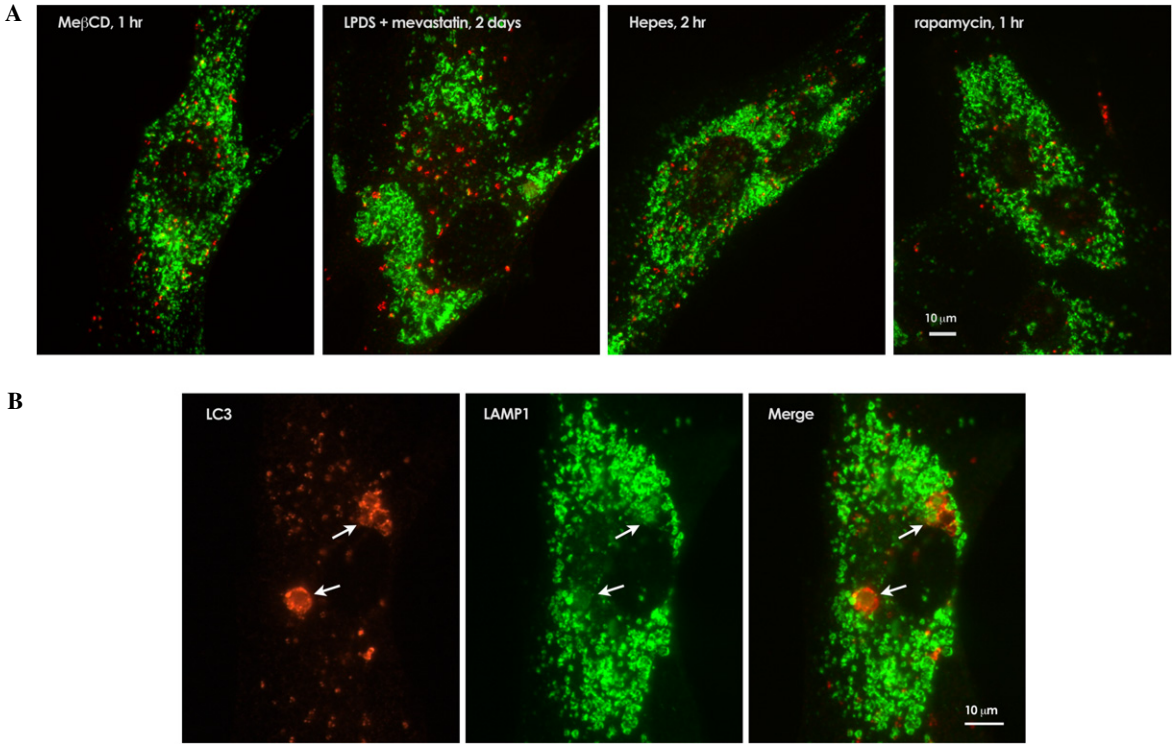

Fig. 4. Double labeling of LC3 and LAMP1. (A) Human fibroblasts were cultured in 5 mM MeβCD for 1 h, 10% LPDS plus mevastatin/mevalonolactone for 3 days, Hanks' solution for 2 h, or 5 μM rapamycin for 1 h, and double-labeled for LC3 (red) and LAMP1 (green). Labeling for LAMP1 was extensive in all the samples, but the overlap with the LC3 labeling was small. (B) Large circular LC3-positive structures observed in cells cultured in 10% LPDS plus mevastatin/mevalonolactone for 3 days, and some of them were weakly labeled for LAMP1 (arrows).

enhanced autophagy may help its degradation, but on the other hand, if reduction of cholesterol retards the maturation of autophagosomes, it may worsen the disease because β-amyloid is generated in the autophagosome [25]. In fact, a recent clinical study showed that an increased level of cholesterol reduces the risk of dementia at old ages [26]. Reduction of cholesterol certainly affects the incidence of Alzheimer's disease, but when and to what extent it is reduced will be critically important in order to obtain the expected results.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.042

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