Bafilomycin A₁ Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion between Autophagosomes and Lysosomes in Rat Hepatoma Cell Line, H-4-II-E Cells

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ABSTRACT. We studied the effects of bafilomycin A₁, a potent and specific inhibitor of vacuolar H⁺ ATPase (V-ATPase), on the process of autophagy in rat hepatoma cell line, H-4-II-E cells. To induce autophagy, cells were transferred from Dulbecco's modified Eagle medium containing 12% fetal calf serum into Hanks' balanced salt solution. When bafilomycin A₁ was added to Hanks' balanced salt solution, endogenous protein degradation was strongly inhibited and numerous autophagosomes accumulated in H-4-II-E cells, whereas autolysosomes decreased in number. Acid phosphatase activity was not detected in the autophagosomes which accumulated in the presence of bafilomycin A₁, suggesting that fusion between autophagosomes and lysosomes was disturbed by this drug. Inhibition of the fusion was reversible, and the autophagosomes changed into autolysosomes after the removal of the inhibitor. Bafilomycin A₁ also prevented the appearance of endocytosed HRP in autophagic vacuoles. These results suggested that acidification of the luminal space of autophagosomes or lysosomes by V-ATPase is important for the fusion between autophagosomes and lysosomes.

Autophagy is one of the main pathways of the degradation of the endogenous proteins and organella (4, 11, 32). In the process of autophagy, membrane structures called isolation membranes or phagophores appear, segregate parts of the cytoplasm, and form autophagosomes. The newly formed autophagosomes (early autophagosomes) fuse with endosomes or prelysosomes, and become a more advanced stage of autophagosomes (late autophagosomes or amphisomes) of acidic luminal pH (2, 11, 12). The autophagosomes then acquire hydrolytic enzymes by fusion with lysosomes, and are transformed into mature autolysosomes in which degradation of the content proceeds (10, 11, 40).

Vacuolar H⁺ ATPase (V-ATPase) is localized in organelles of the central vacuolar system such as coated

vesicles, endosomes, lysosomes, chromaffin granules, and Golgi apparatus, and plays an important role by maintaining the acidic environment in these compartments (for review, see Mellman *et al.*). Bafilomycin A₁, a macrolide antibiotic isolated from *Streptomyces* sp., is a highly specific inhibitor of the V-ATPase (5, 14, 38). Inhibition by bafilomycin A₁ occurs through binding to the membrane-spanning pore forming domain of the enzyme (7, 42). Bafilomycin A₁ was also effective on living cells when added extracellularly (36, 41). Therefore it is a powerful tool to study the role of V-ATPase and vacuolar pH. Using this drug, we have previously reported that V-ATPase is essential for acidifying the lumen of lysosomes and subsequent protein degradation of endocytosed epidermal growth factor (EGF) in lysosomes of cultured cells (41) and phagocytosed rod outer segments in phagolysosomes of retinal pigment epithelial cells (9).

In this study, to clarify the roles of V-ATPase in autophagy, we studied the effects of bafilomycin A₁ on the process of autophagy in rat hepatoma cell line, H-4-II-E cells induced by deprivation of serum and amino acids. Treatment of the cells with bafilomycin A₁ caused accumulation of numerous autophagosomes in the

Abbreviations: V-ATPase, vacuolar H⁺ ATPase; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks' balanced salt solution; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; HRP, horse radish peroxidase.

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cells. These results suggested that acidification of the luminal space of autophagosomes or lysosomes by V-ATPase is important for the fusion between autophagosomes and lysosomes and subsequent degradation of the sequestered cytoplasm in the autolysosomes.

MATERIALS AND METHOD

Materials

Bafilomycin A₁ was kindly provided by Prof. K. Altendorf Universität Osnabrück, Germany). Bafilomycin A₁ was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C . 3-methyl adenine was purchased from Nacalai Tesque Inc, Kyoto, Japan.

Cell culture

A cell line, H-4-II-E-C3 cells (ATCC No. CRL-1600) derived from rat hepatoma H35 cells, was used throughout this study. H-4-II-E cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 12% fetal calf serum (FCS). To induce autophagy, cells were incubated in Hanks' balanced salt solution (HBSS) containing 40 mM Hepes-NaOH buffer pH 7.4. For bafilomycin A₁ treatment experiments, 1/100 volume

of bafilomycin A₁ dissolved in (DMSO) was added to the medium. For control experiments, 1% DMSO was added to the medium.

Measurement of endogenous protein degradation

Protein degradation was measured by the method of Ballard *et al.* (3) with some modifications. H-4-II-E cells were incubated with DMEM containing 12% FCS and 1.25 $\mu\text{Ci}/\text{ml}$ of ^{14}C -leucine for 24 hours at 37°C . After washing with HBSS containing 0.1% bovine serum albumin (BSA) and 40 mM Hepes-NaOH buffer at pH 7.4 three times, cells were incubated with the same medium in the presence of 100 nM bafilomycin A₁, 10 mM NH_4Cl , or 1% DMSO as control. The release of degradation products during incubation was measured by collecting 100 μl of the medium. Proteins were precipitated by 10% (final concentration) TCA, and the supernatant was assayed for radioactivity by liquid scintillation counter after centrifugation. To assess total cell radioactivity, cells were lysed by adding 0.1 M NaOH at the end of the incubation (2 hours), and the radioactivity was measured.

Fluorescence microscopy of vitally stained cells

Cells were incubated for 1 hour at 37°C with DMEM contain-

Fig. 1. Electron micrographs of H-4-II-E cells before and after the induction of autophagy. A. A cell cultured in DMEM containing 12% FCS. Small lysosomes (L) scattered in the cytoplasm. B. A cell transferred into HBSS and incubated for 1 hour. Autophagosomes (AP) and autolysosomes (AL) appeared. Bars; 1 μm .

ing 12% FCS or HBSS in the absence (only 1% DMSO was added at final concentration) or presence of bafilomycin A₁, and vitally stained with 5 $\mu\text{g}/\text{ml}$ acridine orange as described previously (20, 41).

Conventional electron microscopy

H-4-II-E cells in culture dish were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 30 min., washed three times in the same buffer, and post-fixed in 1% OsO₄ in

the same buffer for 1 hour. Cells were dehydrated with a series of ethanol and embedded in epon with the plastic culture dish. After the resin hardened, the plastic dish was removed from the epon block. Ultra-thin sections were cut horizontally to the bottom of the dish, and picked up on collodion-carbon coated grids, doubly stained with uranyl acetate and lead citrate, and observed under a Hitachi H-7000 electron microscope. The area of autophagosomes and autolysosomes was measured on the electron micrographs by a digitizer model SD

Fig. 2. Vital staining of acidic compartments with acridine orange. H-4-II-E cells were cultured in DMEM containing 12% FCS (A, C) or in HBSS (B, D) in the absence (only 1% DMSO) (A, B) or presence of 100 nM bafilomycin A₁ (C, D), and vitally stained with 5 $\mu\text{g}/\text{ml}$ acridine orange. A. Many fine granules appeared in cells cultured in DMEM. B. After being transferred into HBSS for 1 hour, stained granules increased in size. C, D. In both culture conditions, 100 nM bafilomycin A₁ completely diminished the staining. Bars; 20 μm .

331 (Wacom, Saitama, Japan) connected to a PC-9801 CV microcomputer (NEC, Tokyo, Japan).

Cytochemical detection of acid phosphatase at electron microscopic level

H-4-II-E cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 30 min., and were washed three times in the same buffer. Acid phosphatase was detected using β -glycerophosphate as a substrate, and cerium as a capture reagent according to Robinson and Karnovsky (28). Cells were post-fixed in 1% OsO_4 , and embedded in epon as described above.

Endocytosis of horse radish peroxidase

H-4-II-E cells were incubated with 10 mg/ml horse radish peroxidase (HRP) (Type II, Sigma, St. Louis, MO), dissolved in HBSS at 37°C for 1 hour. To visualize internalized HRP, cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 30 min., washed three times in the same buffer, and were incubated in diaminobenzidine and H_2O_2 as described by Marsh *et al.* (21). Cells were post-fixed in 1% OsO_4 , dehydrate, and embedded in epon.

RESULTS

Induction of autophagy in H-4-II-E cells after deprivation of amino acid and serum

When H-4-II-E cells were cultured in DMEM containing 12% FCS, small lysosomes dispersed throughout the cytoplasm. Autophagosomes and autolysosomes were scarcely found in the cytoplasm (Fig. 1A). To induce autophagy, cells were transferred into HBSS which contained neither amino acids nor FCS. When H-4-II-E cells were transferred into HBSS and incubated for 1 hour, autophagosomes which enveloped the apparently intact cytoplasm, and autolysosomes which contained degraded material appeared in the cytoplasm (Fig. 1B). Using this culture cell system, we examined the effects of bafilomycin A_1 on autophagy.

Inhibition of acidification of intracellular organelles by bafilomycin A_1

Acridine orange accumulates in acidic compartments and vitally stains them. Figs. 2 show acridine orange staining of H-4-II-E cells cultured in DMEM and in HBSS. Many fine granules existed in H-4-II-E cells cultured in DMEM (Fig. 2A). They may correspond to lysosomes. After being transferred into HBSS for 1 hour, granules stained with acridine orange increased in size, corresponding to the appearance of autolysosomes (Fig. 2B). In both culture conditions, 100 nM bafilomycin A_1 completely diminished the staining, suggesting that it effectively inhibits acidification of lysosomes and autolysosomes (Figs. 2C and 2D).

Inhibition of endogenous protein degradation by bafilomycin A_1

Next, we tested whether bafilomycin A_1 inhibits endogenous protein degradation in H-4-II-E cells or not. H-4-II-E cells were labeled by ^{14}C -leucine for 24 hours. They were then transferred into HBSS, and the TCA soluble count released into the medium was measured. As shown in Fig. 3, bafilomycin A_1 at the concentration of 100 nM nearly completely inhibited endogenous protein degradation 30 min after its addition. 100 nM Bafilomycin A_1 inhibited the protein degradation more effectively than 10 mM NH_4Cl which is known to inhibit protein degradation by neutralizing acidity of the lysosomal compartment (29, 30).

Ultrastructure of H-4-II-E cells treated with bafilomycin A_1

When H-4-II-E cells were incubated in HBSS in the presence of 100 nM (Fig. 4A) or 1 μM (Fig. 4B) bafilomycin A_1 for 1 hour, numerous autophagosomes appeared in the cytoplasm, but few autolysosomes were seen. Few changes took place in other organelles except for the swelling of Golgi cisterna especially in trans-Golgi

Fig. 3. Inhibition of endogenous protein degradation by bafilomycin A_1 . H-4-II-E cells were labelled in DMEM containing 12% FCS and 1.25 $\mu\text{Ci}/\text{ml}$ of ^{14}C -leucine for 24 hour. Cells were then incubated with HBSS containing 0.1% BSA and 40 mM Hepes-NaOH at pH 7.4 in the presence of 100 nM bafilomycin A_1 (●), 10 mM NH_4Cl (▲) or only 1% DMSO as control (○). Release of degradation products during incubation was measured. Proteins were precipitated by 10% TCA, and TCA soluble radioactivity was measured by liquid scintillation counter. The vertical axis shows the percentage of the total cell count. Means of two experiments are shown.

Fig. 4. Electron micrographs of H-4-II-E cells treated with bafilomycin A₁. A, B. Cells incubated with HBSS containing 100 nM (A) or 1 μ M (B) bafilomycin A₁ for 1 hour. Autophagosomes (AP) accumulated in the cytoplasm. G; Golgi apparatus. C. A cell incubated with DMEM containing 1 μ M bafilomycin A₁ for 1 hour. D. Cells were incubated in HBSS in the presence of 100 nM bafilomycin A₁ for 1 hour, and were transferred into DMEM in the absence of the drug. Autophagosomes have already transformed into autolysosomes. Bars; 1 μ m.

region as observed in GH₃ cells treated with bafilomycin A₁ (15). In DMEM, however, the addition of bafilomycin A₁ did not cause any morphological changes in H-4-II-E cells (Fig. 4C). 3-methyl adenine is well known as a specific inhibitor of autophagy (31). Addition of 10 mM 3-methyl adenine to HBSS inhibited the appearance of autophagosomes and autolysosomes almost completely both in the presence and in the absence of 1 μ M bafilomycin A₁ (data not shown). These results suggested that the accumulation of autophagosomes by bafilomycin A₁ treatment results from the inhibition of maturation of autophagosomes to autolysosomes rather than the acceleration of the formation of autophagosomes.

To determine whether the effect of bafilomycin A₁ is reversible or not, we incubated H-4-II-E cells in HBSS in the presence of 100 nM bafilomycin A₁ for 1 hour, and then transferred the cells into DMEM in the absence of the drug. In DMEM, formation of new phagosomes is strongly suppressed as described above. As a result, the autophagosomes which accumulated in H-4-II-E cells by bafilomycin A₁ treatment transformed into autolysosomes, showing that the effect of bafilomycin A₁

Fig. 5. Kinetics of the appearance of autophagosomes and autolysosomes. H-4-II-E cells were transferred from DMEM containing 12% FCS into HBSS in the absence (Left, only 1% DMSO was added) or in the presence of 100 nM bafilomycin A₁ (Right). The area of profiles of autophagosomes (○) or autolysosomes (●) was measured on electron micrographs.

Fig. 6. Cytochemical demonstration of acid phosphatase activity. A. A H-4-II-E cell incubated with HBSS containing 1% DMSO for 1 hour. Acid phosphatase negative autophagosomes (AP) and acid phosphatase positive autolysosomes (AL) are seen. B. A H-4-II-E cell incubated for 1 hour in HBSS containing 1 μ M bafilomycin A₁ for 1 hour. Acid phosphatase activity was not detected in the autophagosomes (AP) which accumulated in the cytoplasm. Bars; 1 μ m.

is reversible (Fig. 4D).

Kinetics of the appearance of autophagosomes and autolysosomes in bafilomycin A₁ treated and untreated cells

Fig. 5 shows the kinetics of the appearance of autophagosomes and autolysosomes in H-4-II-E cells after serum and amino acids deprivation. For this purpose, the area of autophagosomes or autolysosomes was measured on the electron micrographs. In the absence of bafilomycin A₁ (Fig. 5, Left), autophagosomes appeared first after transfer into HBSS, and reached plateau at 30 min. On the other hand, autolysosomes increased linearly, and occupied about 6% of the cytoplasmic area after 2 hours of incubation. In the presence of 100 nM bafilomycin A₁ (Fig. 5, Right), autophagosomes increased linearly, and occupied about 7% of the cytoplasm after 2 hours of incubation, whereas autolysosomes scarcely appeared within 1 hour of incubation. These results showed that the conversion of autophagosomes to autolysosome was markedly suppressed in the presence of bafilomycin A₁.

Cytochemical and histochemical characterization of autophagosomes accumulated in the presence of bafilomycin A₁

First of all, we examined whether autophagosomes accumulated by bafilomycin A₁-treatment lack lysosomal enzymes or not. For this purpose, we demonstrated acid phosphatase activity at electron microscopic level. Fig. 6A shows a H-4-II-E cell incubated in HBSS for 1 hour. Acid phosphatase activity is seen in autolysosomes but not in autophagosomes. Fig. 6B shows a H-4-II-E cell incubated in HBSS in the presence of 1 μ M bafilomycin A₁ for 1 hour. Autophagosomes that accumulated in the cytoplasm did not show any acid phosphatase activity. These results suggest that fusion between autophagosomes and lysosomes was inhibited in the presence of bafilomycin A₁.

Convergence of autophagic and endocytic pathways has been reported by several investigators (10, 12, 19, 35). Therefore, we examined whether bafilomycin A₁ inhibits this convergence process. For this purpose, H-4-II-E cells were incubated for 1 hour in HBSS containing HRP. HRP was detected in autolysosomes in the ab-

Fig. 7. Localization of HRP internalized by H-4-II-E cells. A. A H-4-II-E cell incubated in HBSS containing 10 mg/ml HRP and 1% DMSO for 1 hour. The reaction products showing the presence of HRP are seen in autolysosomes (AL), but not in autophagosomes (AP). B. A H-4-II-E cell incubated for 1 hour in HBSS containing 10 mg/ml HRP and 1 μ M bafilomycin A₁ for 1 hour. Few reaction products are seen in autophagosomes (AP). The arrow shows tubular profiles of HRP labeled endosomes. Bars; 500 nm.

sence of bafilomycin A₁ (Fig. 7A). However, in the presence of 1 μ M bafilomycin A₁, HRP was not detected in the autophagosomes (Fig. 7B). Tubular profiles of HRP labeled endosomes were occasionally found in the bafilomycin A₁ treated cells as reported by Clague *et al.* (6).

DISCUSSION

Bafilomycin A₁ is a potent and specific inhibitor of V-ATPase. Unlike NH₄Cl (29, 30) or monensin (34) which causes vacuolization of lysosomes or Golgi apparatus, this antibiotic causes very few side effects. That is why this drug is now widely used as a powerful tool to study the role of V-ATPase and acidic compartments.

In this study, we studied the effects of bafilomycin A₁ on the process of autophagy induced in H-4-II-E cells by serum and amino acids deprivation, and found that bafilomycin A₁ prevents maturation of autophagosomes into autolysosomes by inhibiting fusion between autophagosomes and lysosomes, and inhibits endogenous protein degradation effectively. These results suggest that the acidification of the luminal space of autophagosomes or lysosomes by V-ATPase may be very important for the fusion between them.

Interestingly, there are many reports showing that deacidification of acidic compartments by bafilomycin A₁ or another V-ATPase inhibitor, concanamycin (17), inhibits or perturbs vesicular transport of the proteins in trans-Golgi region (15, 39), to lysosomes (24), to vacuole in yeast (18), in recycling of transferrin receptor (25), in recycling to the trans-Golgi network from the plasma membrane (27), from early endosomes to late endosomes (6), and from late endosomes to lysosomes (37).

Gruenberg and his colleagues investigated the inhibition of protein transport from early endosomes to late endosomes by bafilomycin A₁ (1, 6). They showed that β -COP and ϵ -COP associate to endosomes, and this association as well as endosome carrier vesicles formation depends on the luminal acidity, and suggested the existence of a trans-membrane pH sensor (1). It has been shown that autophagosomes become acidic prior to the acquisition of hydrolytic enzymes (2, 11, 12). Therefore, it is possible that fusion between autophagosomes and lysosomes is regulated by the trans-membrane pH sensor which is activated after the luminal pH of autophagosomes is sufficiently lowered. Recently, it was reported that a syntaxin homologue, Vam3p functions in fusion of autophagosomes with the vacuole in yeast cells (8). It will be interesting to investigate whether docking and fusion by the SNARE proteins are regulated by luminal pH in the endosome-lysosomal system.

The results obtained in this study are inconsistent

with those obtained in the case of yeast cells. In yeast cells, treatment with bafilomycin A₁ (33) or the destruction of V-ATPase gene (23) caused inhibition of degradation in the vacuoles, but did not block fusion between autophagosomes and the vacuole. The differences suggest that the fusion of autophagosomes with lysosomes/vacuoles may be regulated by different mechanisms in mammalian cells and yeast cells.

The results obtained in this study are also inconsistent with those obtained by Punnonen *et al.* (26). They reported that the inhibition of acidification by monensin did not prevent the delivery of cathepsin L to autophagosomes in rat fibroblast cells. The reason for such difference is unknown, though it is possible that it might have been caused by the difference in drugs, bafilomycin A₁ vs monensin; or the difference in cells, hepatoma H-4-II-E cells vs fibroblast. H-4-II-E cells scarcely show autophagic activity in the presence of serum and amino acids, probably because these cells have high insulin sensitivity (13, 16). Therefore this culture cell system is suitable for studying the early processes of autophagy such as the formation of autophagosomes or acquisition of hydrolytic enzymes after the induction of autophagy by serum and amino acid deprivation.

Convergence of autophagic and endocytic pathways has been reported by several groups (10, 12, 19, 35). Bafilomycin A₁ also prevented the appearance of endocytosed HRP in autophagic vacuoles. It remains to be solved whether this resulted from the inhibition of fusion between autophagosomes and endosomes or from the inhibition of transport of HRP in endosomes.

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