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Inhibition of Niemann-Pick-Type C1-Like1 by Ezetimibe Activates Autophagy in Human Hepatocyte and Reduces Mutant α1-Antitrypsin Z Deposition

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Autophagy can degrade aggregate-prone proteins, but excessive autophagy can have adverse effects. It would be beneficial if autophagy could be enhanced in a cell type-specific manner, but this has been difficult because the basic mechanism of autophagy is common. In the present study we found that inhibition of Niemann-Pick-type C1-like 1 (NPC1L1) by ezetimibe activates autophagy only in hepatocytes and small intestinal epithelia, but not in other cells. Ezetimibe induced accumulation of free cholesterol in the late endosome/lysosome and increased partitioning of a Ragulator component, LAMTOR1, in rafts. The latter change led to down-regulation of mammalian target of rapamycin (mTOR)C1 activity by decreasing mTOR recruitment to the late endosome/lysosome and activated autophagy. A primary effect of ezetimibe was found to be a decrease of free cholesterol in the plasma membrane, because all the results caused by ezetimibe were suppressed by supplementation of cholesterol as a methyl-β-cyclodextrin complex. By enhancing autophagy in human primary hepatocytes with ezetimibe, insoluble mutant α1-antitrypsin Z was reduced significantly. *Conclusion*: Inhibition of NPC1L1 by ezetimibe activates autophagy in human hepatocytes by modulating cholesterol homeostasis. Ezetimibe may be used to ameliorate liver degeneration in α1-antitrypsin deficiency. (HEPATOLOGY 2013;00:000-000)

Macroautophagy (hereafter referred to as autophagy) is a degradation process that is conserved in all eukaryotes, in which cytoplasmic portions are enclosed within the autophagosome and eventually digested in the lysosome. The primary role of autophagy is to maintain cellular homeostasis against various stresses, but recent studies revealed that autophagy is involved in a wider range of activities.[1]

With regard to diseases, activation of autophagy was shown to reduce potentially toxic protein aggregates.[2,3] It is notable that a lack of constitutive autophagy induced the deposition of protein aggregates and caused neurodegeneration.[1] The results suggest that autophagy activators may be used to treat degenerative diseases.[4] To harness the benefit of autophagy and avoid unwanted side effects to the furthest extent possible, it would be useful if autophagy could be activated in restricted cell types. This may not be an easy task, however, because the basic molecular mechanism for autophagic induction and progression appears to be common in all mammalian cells.

We supposed that cellular cholesterol may be a possible point of manipulation to induce cell type-specific autophagy. In a previous study, we found that cholesterol depletion activates autophagy, suggesting that the

Abbreviations: ATZ, α1-antitrypsin Z; CBZ, carbamazepine; DeLS, delipidated serum; DMEM, Dulbecco's modified Eagle's medium; DRM, detergent-resistant membrane; FC, free cholesterol; FCS, fetal calf serum; LE/Ly, late endosomes/lysosome; LPDS, lipoprotein-deficient serum; MβCD, methyl-β-cyclodextrin; NPC1L1, Niemann-Pick-type C1-like1; S6K, p70 ribosomal S6 kinase.

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J_ID: HEP Customer A_ID: HEP26930 Cadmus Art: HEP26930 Ed. Ref. No.: 13-0332.R2 Date: 14-November-13 Stage: Page: 2

intracellular cholesterol homeostasis is related to autophagic regulation.[5] More recently, cholesterol depletion was shown to increase the expression of autophagy-related genes.[6] To exploit the cholesterol-autophagy connection to manipulate autophagy, we turned to ezetimibe, which is thought to function by inhibiting Niemann-Pick-type C1-like1 (NPC1L1) protein that mediates cholesterol uptake.[7] Because the expression of NPC1L1 in human is confined to hepatocytes and small intestinal epithelia, the effect of ezetimibe should be limited to these cells.

In the present study we found that ezetimibe induced autophagy in human hepatocytes. Ezetimibe caused free cholesterol (FC) accumulation in the late endosomes/lysosome (LE/Ly), which resulted in decreased recruitment of mammalian target of rapamycin (mTOR)C1 to the LE/Ly membrane and autophagic induction. Importantly, we found that ezetimibe reduced the aggregation-prone α1-antitrypsin Z (ATZ) mutant, but not wild-type AT (ATM) and endogenous AT in primary hepatocytes in culture. These results indicated that ezetimibe may be used to treat liver disease in patients with AT deficiency.

Materials and Methods

Please see Supporting Materials and Methods for details.

Cholesterol Manipulation. For cholesterol depletion, cells were treated with methyl-β-cyclodextrin (MβCD) in Dulbecco's modified Eagle's medium (DMEM). For cholesterol supplementation, an MβCD-cholesterol complex (MβCD-FC) was added to the full culture medium.

Transfection. Adenovirus vectors carrying ATZ-V5, ATM-V5, and green fluorescent protein (GFP) complementary DNA (cDNA) were infected to human primary hepatocytes at a multiplicity of infection of 1.5.

Subcellular Fractionation. To analyze the detergent-resistant membrane (DRM), cells were treated with 1% Triton X-100 on ice and subjected to sucrose density-gradient ultracentrifugation. The plasma membrane and the late endosome were isolated for lipid analysis.[8,9]

Western Blotting. An insoluble fraction of cells was obtained by lysing cells in a buffer containing 0.5% Triton X-100 and 0.5% sodium deoxycholate as described.[10]

Microscopy. GM1 were detected by a biotinylated cholera toxin B subunit followed by FITC-Avidin D, and FC was labeled by filipin. For intracellular antigens, cells were permeabilized with either 0.01% digitonin or 0.1% Triton X-100.

Quantification of Lipids. FC and choline were quantified using a Determiner L FC kit and a Determiner PL kit (Kyowa Medex), respectively.

Results

Ezetimibe Induced Autophagy by Suppressing mTORC1. Huh7 cells that retain the ability to secrete low-density lipoproteins were used in most experiments,[11] because it is impractical to use large amounts of human primary cells. As shown in later experiments, however, the effect of ezetimibe was more robustly observed in human primary hepatocytes than in Huh7 cells.

When Huh7 cells were treated with ezetimibe, LC3-II, an authentic marker of autophagic membranes,[12] increased significantly, whereas p62, a protein that is engulfed by autophagosomes,[13] was decreased by ezetimibe treatment (Fig. 1A). The ezetimibe-induced LC3-II increased further after longer treatments (Fig. 1B). LC3-positive dots observed by immunofluorescence microscopy also increased by ezetimibe treatment (Fig. 1C). Electron microscopy confirmed the presence of numerous isolation membranes in cells treated with ezetimibe (Fig. 1D). F1

The increase in LC3-II may be caused by suppression of the autophagic flux rather than activation of autophagy. However, this possibility was rejected because LC3-II increased further by impairing lysosomal acidification with chloroquine[14] (Fig. 1E). Completion of the autophagic flow was also confirmed by a tandem fluorescent-tagged LC3 system[15] (Fig. 1F; Supporting Fig. S1).

To explore the mechanism of autophagic induction, we examined the activity of mTORC1, which plays

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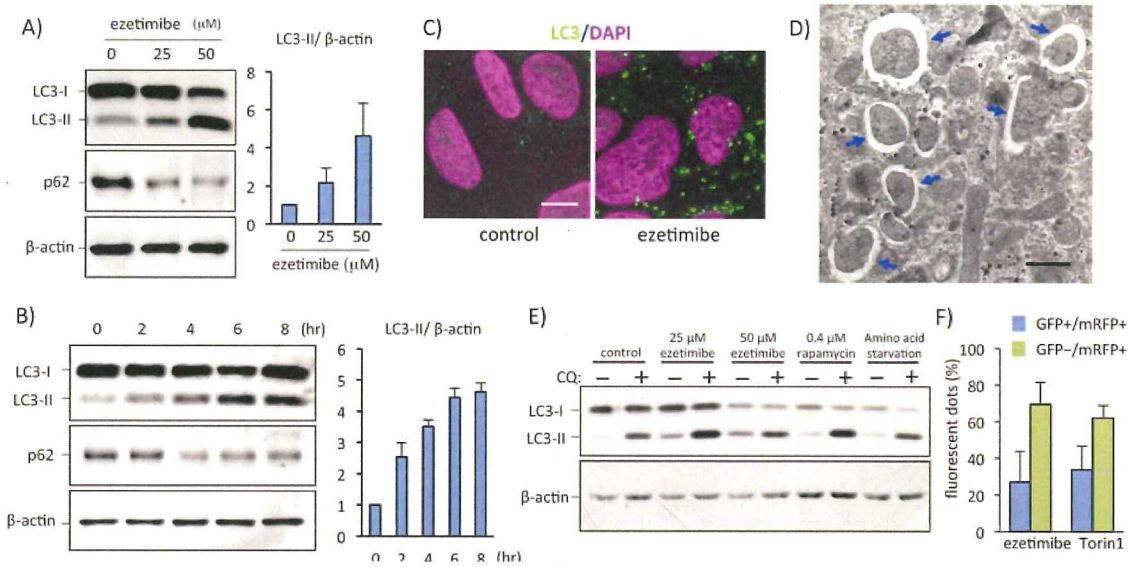

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Fig. 1. Ezetimibe induced autophagy in Huh7 cells. (A) Cells were treated with 25-50 μM ezetimibe for 4 hours. Western blotting shows that ezetimibe caused an increase in LC3-II and a decrease in p62. In this and subsequent figures, a representative western blotting result and a bar graph showing the average band intensity ratio of LC3-II and β-actin obtained from three independent experiments (± SD) are presented. (B) LC3-II and the relative LC3-II/β-actin ratio (bar graph) were increased gradually as a result of treatment with 25 μM ezetimibe. p62 decreased to the lowest level at 4 hours and showed a partial recovery at 6 and 8 hours. (C) LC3-positive dots (green) increased significantly by treating cells with 25 μM ezetimibe for 4 hours. The nuclei were stained with DAPI (magenta). Bar: 10 μm. (D) Cells treated with 25 μM ezetimibe for 4 hours. Isolation membranes were observed frequently by electron microscopy (arrows). Bar: 0.5 μm. (E) Cells were treated with 25-50 μM ezetimibe or 0.4 μM rapamycin for 3 hours and cultured for another 1 hour without or with the addition of 20 μM chloroquine (CQ). Another group of cells was starved of amino acids by culturing in Earle's solution with or without 20 μM chloroquine for 1 hour. The LC3-II increment by chloroquine occurred in comparable degrees in all groups. (F) Cells expressing tandem fluorescent-tagged LC3 were treated with 25 μM ezetimibe or 0.25 μM Torin1 for 4 hours. The number of dots showing mRFP alone and both mRFP and GFP, which indicate mature and immature autolysosomes, respectively, was quantified (n = 5). The relative ratio of the two kinds of dots did not differ between the two samples.

essential roles in the regulation of autophagy.[16] Ezetimibe decreased phosphorylation of p70 ribosomal S6 kinase (S6K), an mTORC1 substrate, in a dose- and F2 time-dependent manner (Fig. 2A,B). The result indicated that ezetimibe induced autophagy by down-regulating the mTORC1 activity.

Decrease in FC in the Plasma/Endosomal Membranes Was Critical for the Ezetimibe Effect. FC depletion was shown to suppress mTORC1 and induce autophagy.[5] Because ezetimibe inhibits FC uptake through NPC1L1,[7,17] the autophagic induction by ezetimibe may also be caused by FC depletion. However, treatment with ezetimibe did not decrease the total cellular FC content, but increased it F3 (Fig. 3A). This contrasted with the significant FC decrease following FC extraction with MβCD or treatment with statin in a lipoprotein-deficient medium (Fig. 3A). Nonetheless, an increase in LC3-II was more evident in cells treated with ezetimibe than in cells treated with statin in a lipoprotein-deficient medium (Fig. 3B). The result showed that ezetimibe induced autophagy without decreasing the total FC content.

To further explore effects of ezetimibe, the FC distribution was visualized with filipin. After ezetimibe treatment, intense filipin labeling was observed in LE/Ly (Fig. 3C), as in NPC1-deficient cells, suggesting that FC efflux from LE/Ly was perturbed. The FC deposition in LE/Ly was not observed when the ezetimibe treatment was done in the delipidated serum (DeLS), but an increase in LC3-II and a decrease in phosphorylated S6K were observed (Fig. S2). The result indicated that the FC deposition in LE/Ly is not the cause of autophagy but a result of some upstream event.

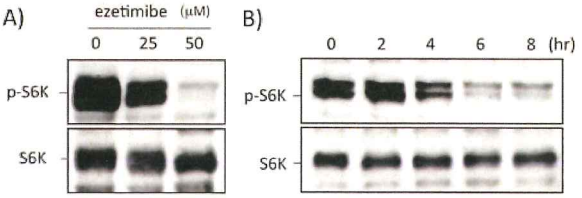

Fig. 2. Ezetimibe suppressed mTORC1 activity in Huh7 cells. (A) Cells were treated with 25-50 μM ezetimibe for 4 hours. Phosphorylated S6K, but not total S6K, decreased significantly. (B) The relative ratio of phosphorylated to total S6K decreased time-dependently in cells treated with 25 μM ezetimibe.

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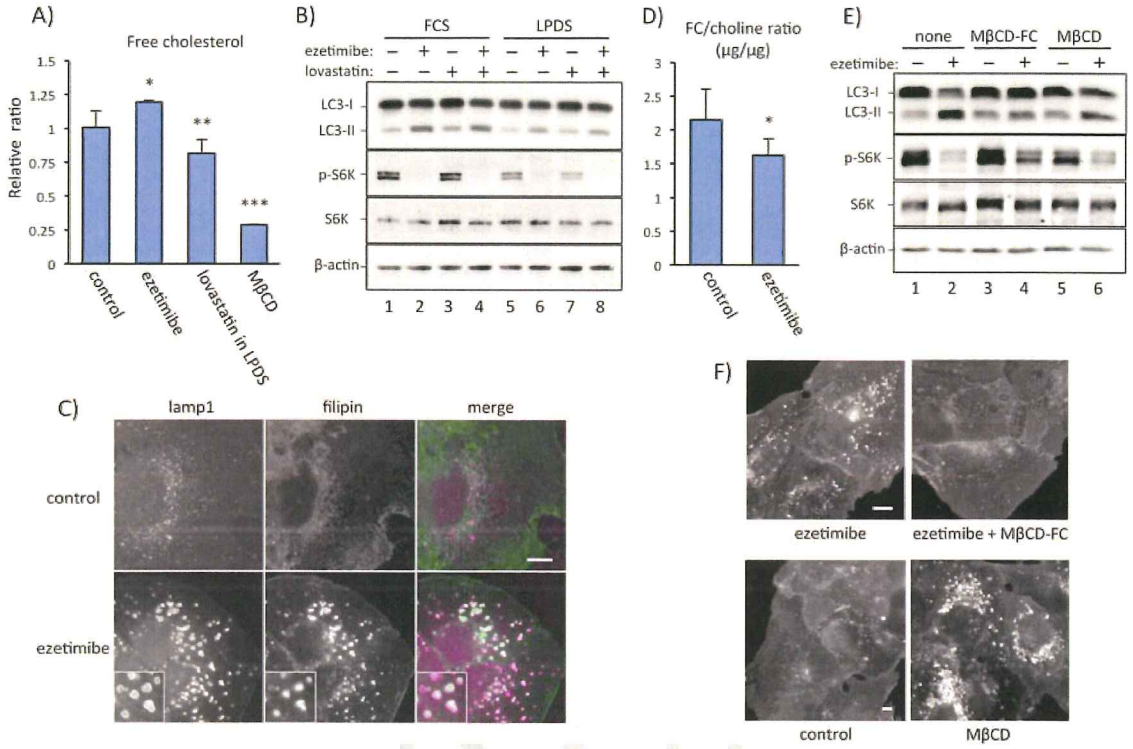

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Fig. 3. The decrease in FC in the plasma/endosomal membranes was critical for autophagy and FC deposition in LE/Ly in ezetimibe-treated Huh7 cells. (A) FC quantification. Cells were treated with either 25 μM ezetimibe in the normal medium for 6 hours, 2 μM lovastatin and 0.25 mM mevalonolactone in 2% lipoprotein-deficient serum (LPDS) for 6 hours, or 5 mM MβCD for 1 hour. Lovastatin/mevalonolactone and MβCD decreased FC, whereas ezetimibe increased FC significantly in comparison to the untreated control (n = 3; $*P = 0.03$, $**P = 0.05$, $***P = 0.0002$). (B) Cells were treated for 4 hours with 25 μM ezetimibe or 2 μM lovastatin/0.25 mM mevalonolactone, in DMEM supplemented with either 10% fetal calf serum (FCS) or 2% LPDS. Ezetimibe in FCS (lane 2) induced an increase in LC3-II and a decrease in phosphorylated S6K more significantly than lovastatin/mevalonolactone alone (lane 3), LPDS alone (lane 5), or their combination (lane 7). (C) Double labeling of filipin (green) and lamp1 (magenta). Cells were treated with 25 μM ezetimibe for 4 hours. Ezetimibe induced FC deposition in the lamp1-positive LE/Ly. Bar: 10 μm. Inset: high magnification. (D) The plasma membrane was isolated from cells untreated or treated with 25 μM ezetimibe for 5 hours by the silica-coating method. FC and choline contents in the extracted lipid were measured in quadruplicate samples. The ratio of FC to choline (representing phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) was decreased significantly by ezetimibe (n = 3; $*P = 0.04$). (E) Cells were treated for 4 hours with 25 μM ezetimibe alone (lane 2) or together with either 0.5 mM MβCD-FC (corresponding to ~100 μg/mL cholesterol) (lane 4) or 0.5 mM MβCD (lane 6). All treatments were carried out in DMEM with 10% FCS. MβCD-FC, but not MβCD, attenuated the increase in LC3-II and the decrease in phosphorylated S6K induced by ezetimibe. (F) (upper figures) FC deposition in LE/Ly was reduced significantly when 0.25 mM MβCD-FC was given with 25 μM ezetimibe for 4 hours. (lower figures) FC deposition in LE/Ly was observed when cells were treated with 0.4 mM MβCD in DMEM for 30 minutes to extract FC mildly. The FC deposition was not observed when the treatment was extended longer (>45 min) or used a higher concentration (>1 mM) of MβCD, which decreased FC excessively. Bars: 10 μm.

Ezetimibe is thought to prevent the conformational change of NPC1L1,[18,19] which is necessary for NPC1L1 to insert FC into the membrane.[20,21] Consistently, FC in the plasma membrane decreased by ezetimibe (Figs. 3D; S3). As such, we thought that the primary effect of ezetimibe is to decrease FC in the membrane where NPC1L1 resides, i.e., the plasma membrane, and the early and recycling endosomal membranes (hereafter called the plasma/endosomal membranes). We thus hypothesized that FC supplementation to the plasma/endosomal membranes with MβCD-FC[22] should attenuate the effects of ezetimibe. In fact, the MβCD-FC treatment increased FC in the plasma/endosomal membranes (Fig. S4) and suppressed the increase in LC3-II and the dephosphorylation of S6K, whereas MβCD alone did not modulate the ezetimibe effect (Fig. 3E).

Furthermore, paradoxically, the FC deposition in LE/Ly induced by ezetimibe was decreased in the presence of MβCD-FC (Figs. 3F; S4). Moreover, FC extraction with a low concentration of MβCD induced FC deposition in LE/Ly (Fig. 3F). These results demonstrated that ezetimibe primarily affected FC in the plasma/endosomal membranes, and that this change led to the aberrant FC deposition in LE/Ly and autophagy.

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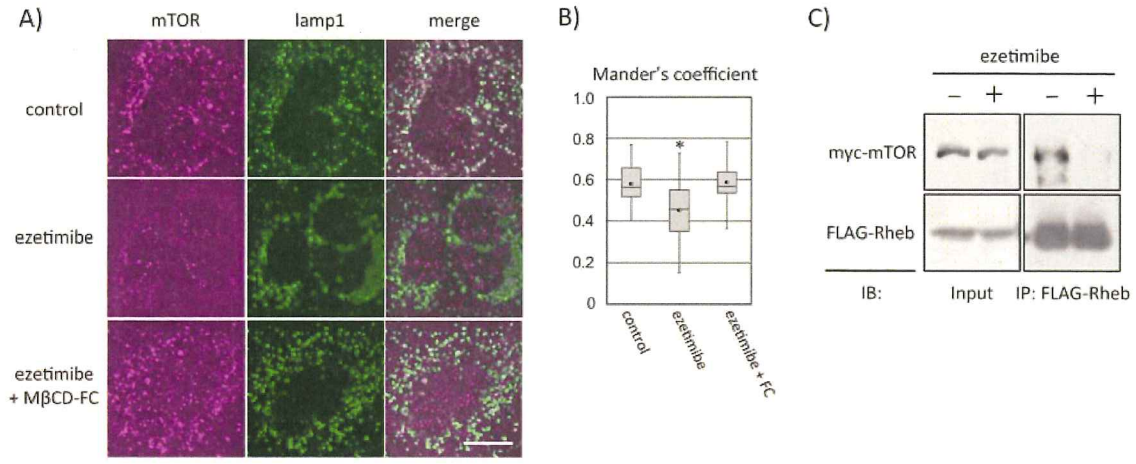

Fig. 4. Ezetimibe decreased mTOR recruitment to LE/Ly. (A) Double labeling of mTOR (magenta) and lamp1 (green). Huh7 cells were treated with 25 μM ezetimibe alone or together with 0.25 mM MβCD-FC for 4 hours. In untreated cells, mTOR showed distinct colocalization with lamp1, but ezetimibe obliterated the colocalization with lamp1. The change by ezetimibe was not observed when ezetimibe and MβCD-FC were added simultaneously. The perinuclear lamp1 distribution did not change by any treatment. Bar: 10 μm. (B) Colocalization analysis of mTOR and lamp1 by thresholded Mander's coefficient. Medians and the 25th and 75th percentiles are shown in box plots. Zero and one indicate no and perfect colocalization, respectively. The coefficient of ezetimibe-treated cells was significantly lower than that of the control or cells treated with ezetimibe and MβCD-FC (n = 19 (control); 36 (ezetimibe); 39 (ezetimibe + FC); *$P = 0.0003$). (C) Coimmunoprecipitation of mTOR and Rheb. Myc-mTOR cosedimenting with FLAG-Rheb decreased by the treatment with 25 μM ezetimibe for 4 hours.

Ezetimibe Reduced Recruitment of mTOR to LE/Ly. In the presence of amino acids, mTORC1 is translocated to LE/Ly, where it is bound and activated by existing Rheb GTPase.[23] We hypothesized that ezetimibe disturbed this process by modulating cholesterol trafficking and thereby suppressed the mTORC1 activity. To test this idea, we first examined the distribution of mTORC1. Immunofluorescence microscopy showed that colocalization of mTOR and lamp1[24] decreased F4 after ezetimibe treatment (Fig. 4A,B). But the change was not observed when the ezetimibe treatment was done in the presence of MβCD-FC (Fig. 4A,B).

The decrease in mTOR in LE/Ly was likely to down-regulate the interaction between mTOR and Rheb. In fact, myc-mTOR coimmunoprecipitating with FLAG-Rheb decreased significantly when cells were treated with ezetimibe (Fig. 4C). The results showed that ezetimibe suppressed mTORC1 activity by decreasing the mTOR-Rheb binding in the LE/Ly membrane.

The recruitment of mTORC1 to LE/Ly is facilitated by Rag-GTPases and the Ragulator complex.[23] Because Ragulator is anchored to the LE/Ly membrane through myristoylation and palmitoylation of LAMTOR1,[25] we suspected that ezetimibe affected the Ragulator functionality by changing the membrane property. In fact, ezetimibe did not affect the immunofluorescence distribution of LAMTOR1 (Fig. S5), but significantly increased the proportion of LAMTOR1 F5 in DRM (Fig. 5A). Flotillin-1, a raft molecule distributed in LE/Ly in Huh7 cells (Fig. S6), also exhibited an increase in DRM by ezetimibe treatment (Fig. 5A). When ezetimibe and MβCD-FC were applied simultaneously, the shift of LAMTOR1 and flotillin-1 from non-DRM to DRM was not observed (Fig. 5B). In contrast, LAMTOR1 increased in non-DRM when FC was depleted with MβCD (Fig. 5A). These results indicated that ezetimibe caused an excessive concentration of Ragulator in the LE/Ly membrane raft. This change probably abrogated the Ragulator functionality and reduced the mTORC1 recruitment to LE/Ly.

Another raft molecule, ganglioside GM1, is distributed largely in the plasma/endosomal membranes (Fig. S7A). GM1 in control cells was recovered in DRM almost exclusively, but after ezetimibe treatment a significant portion was found in non-DRM fractions (Fig. S7B), which was in contrast with the behavior of LAMTOR1 and flotillin-1. The shift of GM1 from DRM to non-DRM did not occur in cells treated with ezetimibe and MβCD-FC simultaneously, indicating that this change was also caused by a decrease in FC in the plasma membrane (Fig. S7B). The results indicated that ezetimibe exerted opposite effects on rafts in the plasma/endosomal membranes and those in the LE/Ly membrane.

***Ezetimibe Induced Autophagy in Mouse Small Intestine* In Vivo.** The effect of ezetimibe in cells *in vivo* was examined using mouse small intestinal epithelium, which shows the highest expression of NPC1L1 in rodents.[17] The intestinal mucosa of mice with or without

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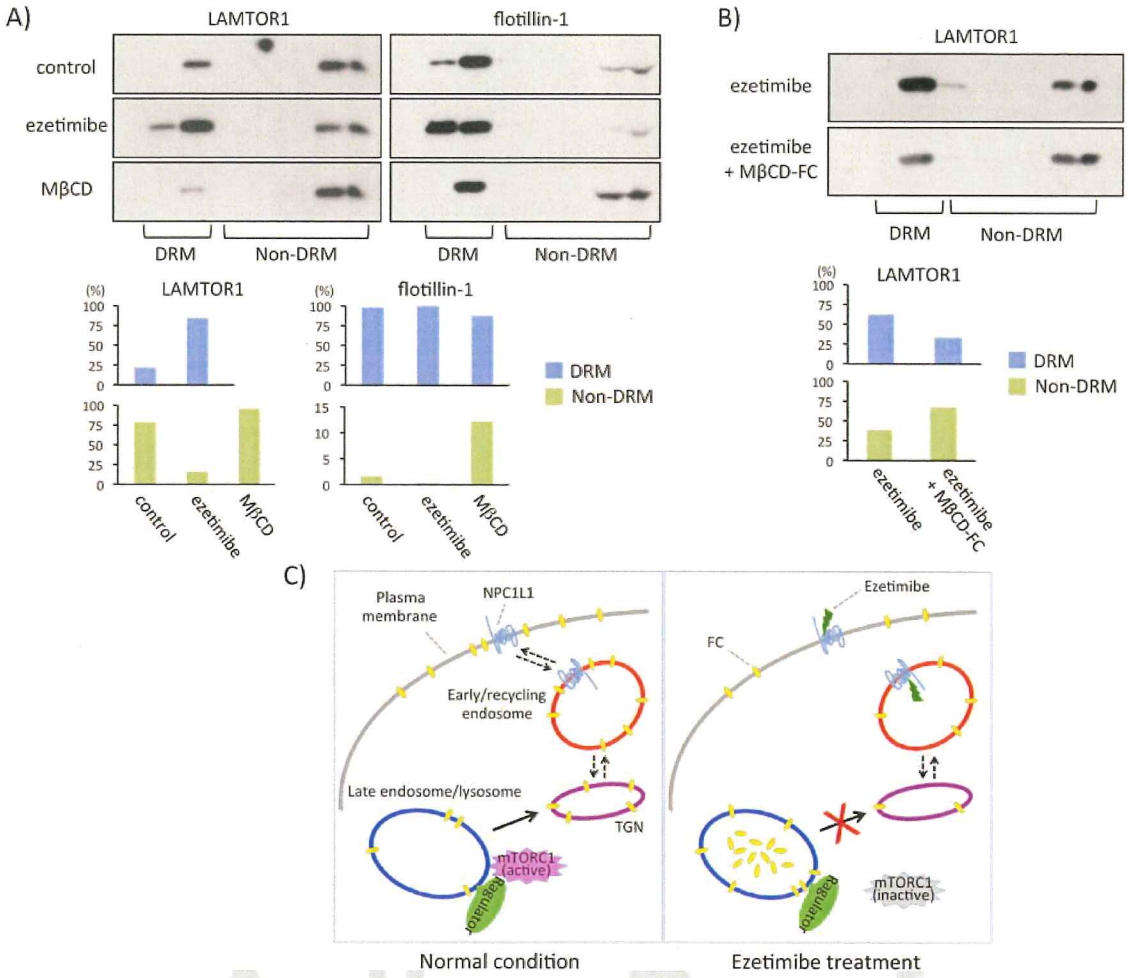

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Fig. 5. Ezetimibe increased LAMTOR1 and flotillin-1 in rafts in Huh7 cells. (A) Cells either left untreated, treated with 25 μM ezetimibe for 4 hours, or treated with 5 mM MβCD in DMEM for 60 minutes were lysed in cold 1% Triton X-100 and separated by sucrose density-gradient centrifugation. The proportion of LAMTOR1 in DRM was drastically increased by ezetimibe, but decreased by MβCD. Flotillin-1 showed a less prominent but similar change. The relative band intensity in DRM and non-DRM fractions is shown in the graph. (B) Cells were treated with 25 μM ezetimibe alone or together with 0.25 mM MβCD-FC for 4 hours and examined as in (A). The increase of LAMTOR1 in DRM was not observed when ezetimibe was applied in the presence of MβCD-FC. (C) The putative mechanism of ezetimibe-induced autophagy. In the normal condition (left), Ragulator in the LE/Ly membrane functions to recruit and activate mTORC1. Upon ezetimibe treatment (right), due to NPC1L1 inhibition, an active pool of FC decreases in the plasma/endosomal membranes and TGN. This in turn perturbs the FC export from LE/Ly, induces excessive raft formation in the LE/Ly membrane, and compromises the Ragulator functionality.

ezetimibe administered by gavage were compared. By western blotting (Fig. 6A), the increase in LC3-II was relatively small, most likely because the mucosal sample contained other submucosal tissues besides epithelial cells, yet immunofluorescence microscopy showed that LC3-positive dots in the epithelial cell increased significantly following the ezetimibe treatment (Fig. 6B). The increase in autophagolysosomes was confirmed by electron microscopy (Fig. 6C). These results indicate that ezetimibe can induce autophagy in cells *in vivo*.

Ezetimibe Reduced Mutant α1-Antitrypsin in Human Primary Hepatocytes. ATZ forms insoluble aggregates in the endoplasmic reticulum (ER) lumen of hepatocytes and causes serious liver damage.[26] Because autophagy was shown to degrade ATZ,[10,27] we examined if ezetimibe can reduce ATZ by activating autophagy in primary human hepatocytes. By treating cells with 25 μM ezetimibe, LC3-II increased, whereas phosphorylated S6K and p62 decreased significantly (Fig. 7A), confirming that ezetimibe enhances autophagy in nontransformed human hepatocytes.

Next, human hepatocytes expressing ATZ were treated with ezetimibe for 1-2 days, and an increase in LC3-II was induced without suppression of the autophagic flux (Fig. S8). After ezetimibe treatment, both the total cellular ATZ and the insoluble ATZ decreased

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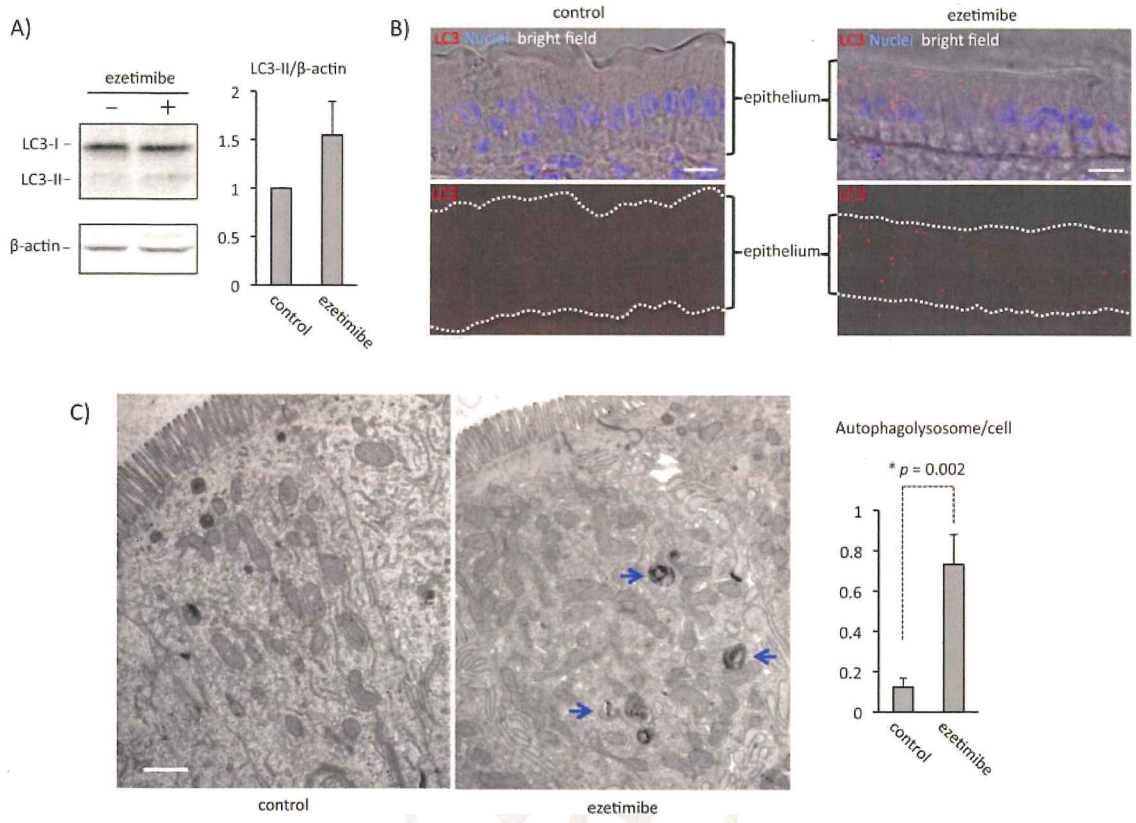

Fig. 6. Ezetimibe induced autophagy in the mouse small intestinal mucosa *in vivo*. Mice were administered 10 μg ezetimibe/g body weight by gavage, and the small intestinal mucosa were examined 4 hours later. (A) Ezetimibe induced an increase in LC3-II. (B) Immunofluorescence microscopy showed that LC3-positive dots (red) increased significantly in the epithelium following ezetimibe treatment. Nuclei are stained blue by Hoechst 33342. Bars: 10 μm. (C) Electron microscopy confirmed the increase of autophagolysosomes (arrows) in the ezetimibe-treated epithelium. Bar: 0.5 μm. Autophagolysosomes in more than 100 epithelial cells were counted in electron micrographs.

to a degree comparable to or even larger than carbamazepine (CBZ), a reagent shown to reduce ATZ by enhancing autophagy (Fig. 7B).[10] Because GFP was not decreased by ezetimibe treatment, the decrease in ATZ was not likely to be caused by the general suppression of protein synthesis or other nonspecific mechanisms (Fig. 7B). The ezetimibe treatment drastically decreased mutant ATZ in both the total lysate and the insoluble pellet, whereas it decreased wild-type ATM to a much lesser extent and did not affect endogenous AT at all (Fig. 7C). The result indicated that the effect of ezetimibe was selective to aggregate-prone ATZ.

In contrast to the effect on hepatocytes, ezetimibe did not induce autophagy or suppress the mTORC1 activity in other nontransformed human cells. Human primary fibroblasts and lymphocytes did not show an increase in LC3-II or a decrease in phosphorylated S6K after ezetimibe treatment (Fig. S9). The cell lines of other species that do not express NPC1L1, such as Cos7, did not show autophagic induction either.

Discussion

The present study showed that ezetimibe induces autophagy by perturbing the intracellular cholesterol homeostasis. The linkage between cholesterol and autophagy has been indicated,[5,6,28] but the underlying mechanism remained unclear. We found that the primary effect of ezetimibe is a decrease of FC in the plasma/endosomal membrane, because FC supplementation with MβCD-FC suppressed the changes induced by ezetimibe. The FC decrease in the plasma/endosomal membrane eventually led to an increased partitioning of LAMTOR1 in DRM in the LE/Ly membrane, probably by an FC increase in this membrane, and suppression of the mTORC1 activity. This appears opposite to the change occurring in cells depleted of FC, in which rafts in the LE/Ly membrane were disintegrated and LAMTOR1 in DRM decreased. However, LE/Ly functions are likely to be impaired not only by raft disintegration but also by excessive raft formation.[29,30] It is plausible to think that either

J_ID: HEP Customer A_ID: HEP26930 Cadmus Art: HEP26930 Ed. Ref. No.: 13-0332.R2 Date: 14-November-13 Stage: Page: 8

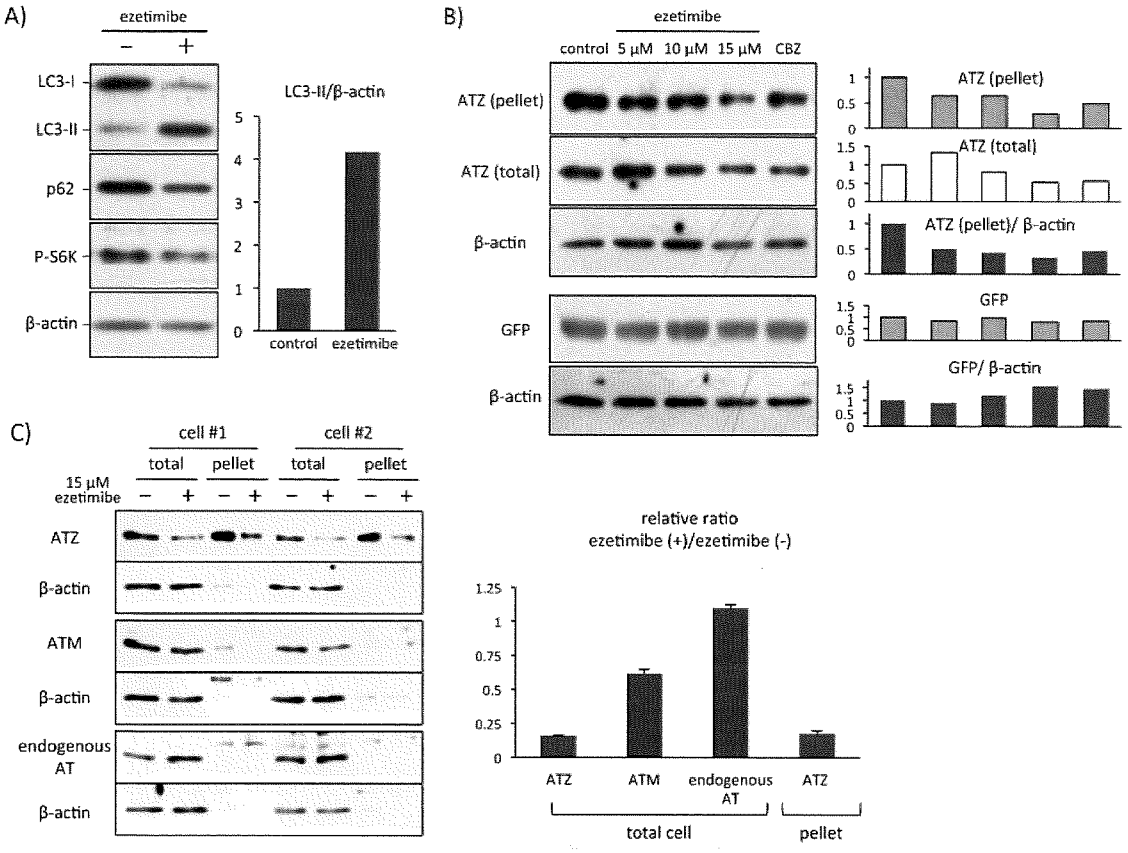

Fig. 7. Ezetimibe induced autophagy and decreased ATZ in human primary hepatocytes. (A) Cells were treated with 25 μM ezetimibe for 4 hours. Ezetimibe induced a significant increase in LC3-II and a decrease in p62 and phosphorylated S6K. (B) Cells were infected with an adenovirus vector carrying ATZ-V5 or GFP at the same multiplicity of infection and treated with 5-15 μM ezetimibe or 25 μM CBZ for 2 days. Both the total lysate and the insoluble fraction were examined for ATZ-V5. The bar graph shows the relative intensity of the ATZ-V5 band and its ratio to β-actin, taking the control sample as the standard. The expression level of ATZ-V5, but not that of GFP, decreased with either ezetimibe or CBZ. (C) Two cell samples were infected with an adenovirus vector carrying ATZ-V5 or ATM-V5 or kept uninfected, and then treated with 15 μM ezetimibe for 1 day. ATZ-V5, ATM-V5, and endogenous AT in the total lysate and the insoluble pellet fraction were examined. The bar graph shows a relative band intensity taking β-actin as a standard. The ezetimibe drastically decreased ATZ-V5 in both the total lysate and the pellet, but the effect on ATM-V5 and endogenous AT was much less. Note that only ATZ-V5 was contained in the insoluble fraction.

an increase or a decrease in FC in the LE/Ly membrane beyond a physiological range causes abnormality by affecting the raft functionality. In ezetimibe-treated cells, excessive rafts in the LE/Ly membrane are thought to suppress mTORC1 activation by compromising the molecular interaction between Ragulator and its binding partners.

The decrease of FC in the plasma/endosomal membrane caused by ezetimibe is relatively mild, but we speculate that FC inserted into the membrane by NPC1L1 may be "active cholesterol,"[31] and that ezetimibe decreased this population of FC preferentially and modulated the cellular FC homeostasis considerably. This probably led to suppression of the vesicular transport of FC from LE/Ly to the trans-Golgi network and caused FC accumulation in LE/Ly.[32,33] The regulatory mechanism to control FC concentration in the LE/Ly membrane has not yet been defined, especially in differentiated cells such as hepatocytes,[34,35] but it is apparently critically important for the induction of autophagy. It would be interesting to study whether some disorders and reagents affect autophagy by perturbing the FC trafficking around LE/Ly.

For AT deficiency, autophagy enhancement by CBZ was shown to reduce the mutant ATZ aggregate in mouse liver.[10] The present study showed that ezetimibe also decreases the ATZ load in hepatocytes. An important difference between ezetimibe and CBZ is that ezetimibe activates autophagy only in cells expressing NPC1L1, so that cells other than hepatocytes and small intestinal epithelial cells would be spared. This property may be beneficial because excessive autophagy might

exert detrimental effects.[1] Moreover, ezetimibe has been used as an anticholesterolemic drug and side effects were reported to be rare and mild.[36] Further basic research remains to be done to reach the clinical level, but the present result indicates that ezetimibe has potential in the treatment of AT-deficiency patients.

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