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Sucrose Induces Vesicle Accumulation and Autophagy

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ABSTRACT

It has been shown that the treatment of mammalian cells with sucrose leads to vacuole accumulation associated with lysosomes and upregulation of lysosomal enzyme expression and activity. Autophagy is an evolutionarily conserved homeostatic process by which cells deliver cytoplasmic material for degradation into lysosomes, thus it is probable that sucrose affects the autophagic activity. The role of sucrose in autophagy is unknown; however, another disaccharide, trehalose has been shown to induce autophagy. In the current study, we used mouse embryonic fibroblasts to investigate whether sucrose induces autophagy and whether vesicle formation is associated with autophagy. The results showed that sucrose induces autophagy while being accumulated within the endosomes/lysosomes. These vesicles were swollen and packed within the cytoplasm. Furthermore, trehalose and the trisaccharide raffinose, which are not hydrolyzed in mammalian cells, increased the rate of vesicles accumulation and LC3-II level (a protein marker of autophagy). However, fructose and maltose did not show the same effects. The correlation between the two processes, vesicle accumulation and autophagy induction, was confirmed by treatment of cells with sucrose plus invertase, or maltose plus acarbose—the α-glucosidase inhibitor—and by sucrose deprivation. Results also showed that vesicle accumulation was not affected by autophagy inhibition. Therefore, the data suggest that sucrose-induced autophagy through accumulation of sucrose-containing vesicles is caused by the absence of hydrolysis enzymes. J. Cell. Biochem. 116: 609–617, 2015.

KEY WORDS: SUCROSE; VESICLE ACCUMULATION; AUTOPHAGY

Sucrose is a neutral disaccharide composed of glucose and fructose. It can be internalized by cells via pinocytosis, but cannot be hydrolyzed in mammalian cells due to absence of hydrolase. It has been shown that treatment of mammalian cells with sucrose leads to the accumulation of lysosome-associated vacuoles [Cohn and Ehrenreich, 1969; Nyberg and Dingle, 1970; Wagner et al., 1971; DeCourcy and Storrie, 1991], and that the resulting phenotype of sucrose-treated cells is similar to the one observed in the lysosomal storage disease [Karageorgos et al.,1997]. Sucrose also upregulates the expression and activities of lysosomal enzymes (Warburton and Wynn, 1976; Kato et al., 1982; Karageorgos et al., 1997; Helip-Wooley and Thoene, 2004). Therefore, loading of sucrose into cells has been used to study endocytosis or lysosomal activity [Kato et al., 1984; Bright et al., 1997; Karageorgos et al., 1997; Bright et al., 2001; Ulloa and Real, 2003].

Autophagy is an evolutionarily conserved homeostatic process by which cells deliver cytoplasmic material for degradation into lysosomes. Expression of the transcription factor C/EBP is upregulated by sucrose treatment (Helip-Wooley and Thoene, 2004), and C/EBPβ was shown to activate autophagy-related (Atg) genes [Yuk et al., 2009; Yang et al., 2014]. Therefore, it is probable that sucrose affects the autophagic activity. However, sucrose's involvement in autophagy has not been reported, although the disaccharide trehalose was shown to induce autophagy [Sarkar et al., 2007; Aguib et al., 2009]. Trehalose is a disaccharide of glucose that can be found in bacteria, plants, and invertebrates in high concentrations and is not hydrolyzed in mammalian cells.

In this study, we examined whether sucrose induces autophagy and whether autophagy is associated with vesicle formation. The results suggested that sucrose causes the accumulation of sucrose-containing vesicles due to the absence of hydrolytic enzymes, thereby inducing autophagy.

MATERIALS AND METHODS

CHEMICALS

Sucrose, maltose, trehalose, raffinose, fructose, yeast invertase and staurosporine were purchased from Wako Pure Chemical Industries,

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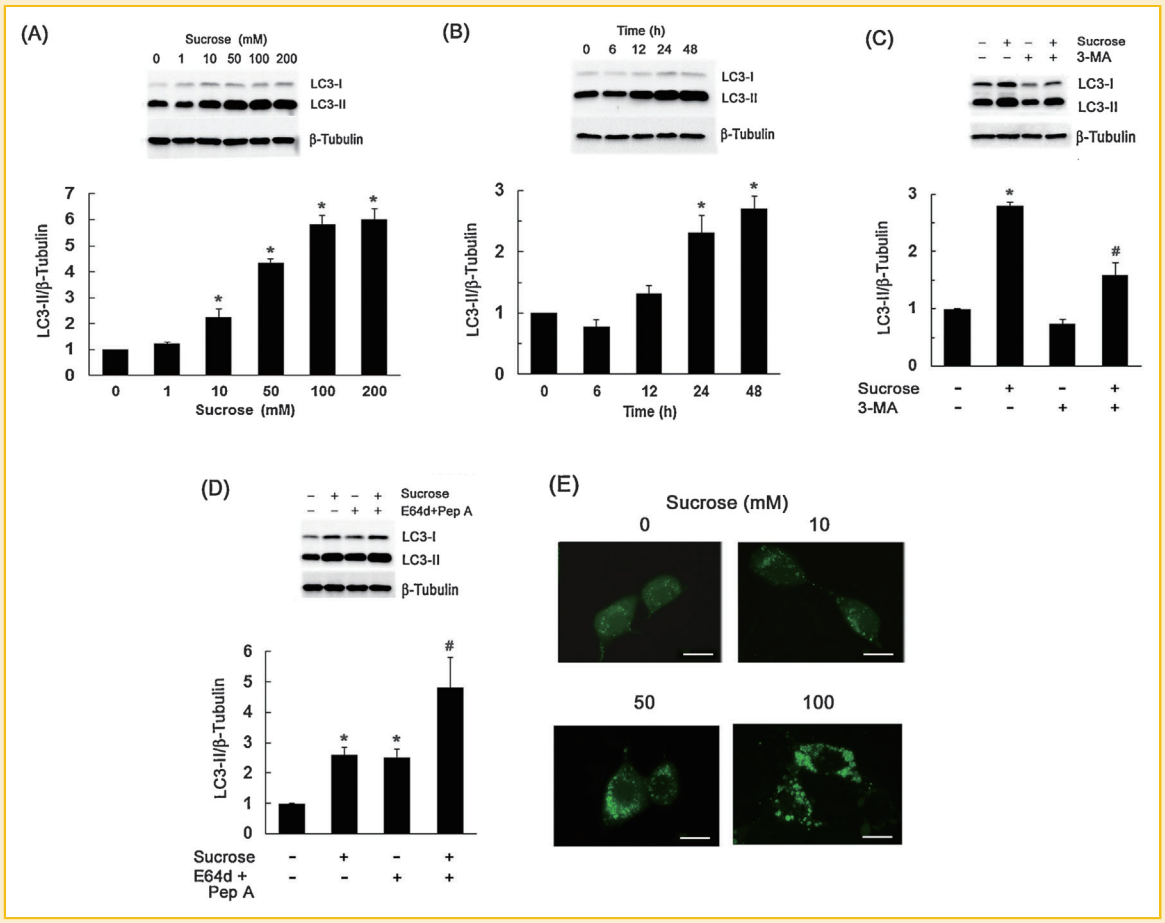

Fig. 1. Sucrose-induced autophagy. (A) MEFs were incubated with the indicated concentration of sucrose for 24 h and LC3-II expression was analyzed. $^{*}P < 0.05$ versus 0. (B) MEFs were incubated with 50 mM sucrose for the indicated times and LC3-II expression was analyzed. $^{*}P < 0.05$ versus 0. (C) MEFs were pretreated with 3-MA (10 mM), incubated with 50 mM sucrose for 24 h, and then LC3-II expression was analyzed. $^{*}P < 0.05$ versus without treatment; $^{\#}P < 0.05$ versus sucrose alone. (D) MEFs were pretreated with lysosomal inhibitors E64d (10 μg/mL) and pepstatin A (Pep A, 10 μg/mL), incubated with 50 mM sucrose for 24 h, and then LC3-II expression was analyzed. $^{*}P < 0.05$ versus without treatment; $^{\#}P < 0.05$ versus with E64d and pepstatin A. The band densities were normalized to those of the untreated cells. (E) MEFs transfected with GFP-LC3 were incubated with the indicated concentration of sucrose for 24 h, and the GFP signals were analyzed by fluorescence microscopy. Bar: 20 μm.

Ltd. (Osaka, Japan). 3-methyladenine, (2S, 3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d) and Nα-benzoyl-DL-arginine-β-naphthylamide (BANA) were purchased from Sigma–Aldrich Co. LTD (St. Louis, MO). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from AppliChem (Darmstadt, Germany). Pepstatin A was purchased from Peptide Institute, Inc. (Osaka, Japan). Acarbose was purchased from LKT laboratories, Inc. (St. Paul, MN). FM4–64 was purchased from Biotium, Inc. (Hayward, CA). Anti-LC3, anti cleaved caspase-3, anti-S6 kinase 1 (S6K1), anti-phospho-S6K1 (Thr389) and anti-β-tubulin antibodies were acquired from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody and LysoTracker Red DND-99 were obtained from Life Technologies Corporation (Grand Island, NY). Lucifer yellow CH dilithium salt was purchased from Sanguine BioSciences, Inc. (Valencia, CA). [^{14}C(U)]-sucrose (435 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

CELL CULTURE AND TREATMENT

Mouse embryonic fibroblasts (MEFs) and Atg gene 5 (Atg5)-deficient MEFs (Atg5$^{-/-}$) [Kuma et al., 2004] were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Human neuroblastoma SH SY5Y cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in DMEM containing 10% FBS at 37 °C in a 5% CO_2 incubator. For experiments, the cells were seeded in culture dishes or plates and grown for 1–2 days, following incubation with sucrose for 24 h. Saccharides were dissolved in the incubation medium. For pretreatment, the cells were incubated for 30–60 min with reagents or vehicles. GFP-LC3 was expressed as previously described [Kabuta et al., 2006].

WESTERN BLOT ANALYSIS

Proteins were extracted from cells, and their concentrations were determined using a protein-assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of proteins were separated on SDS-

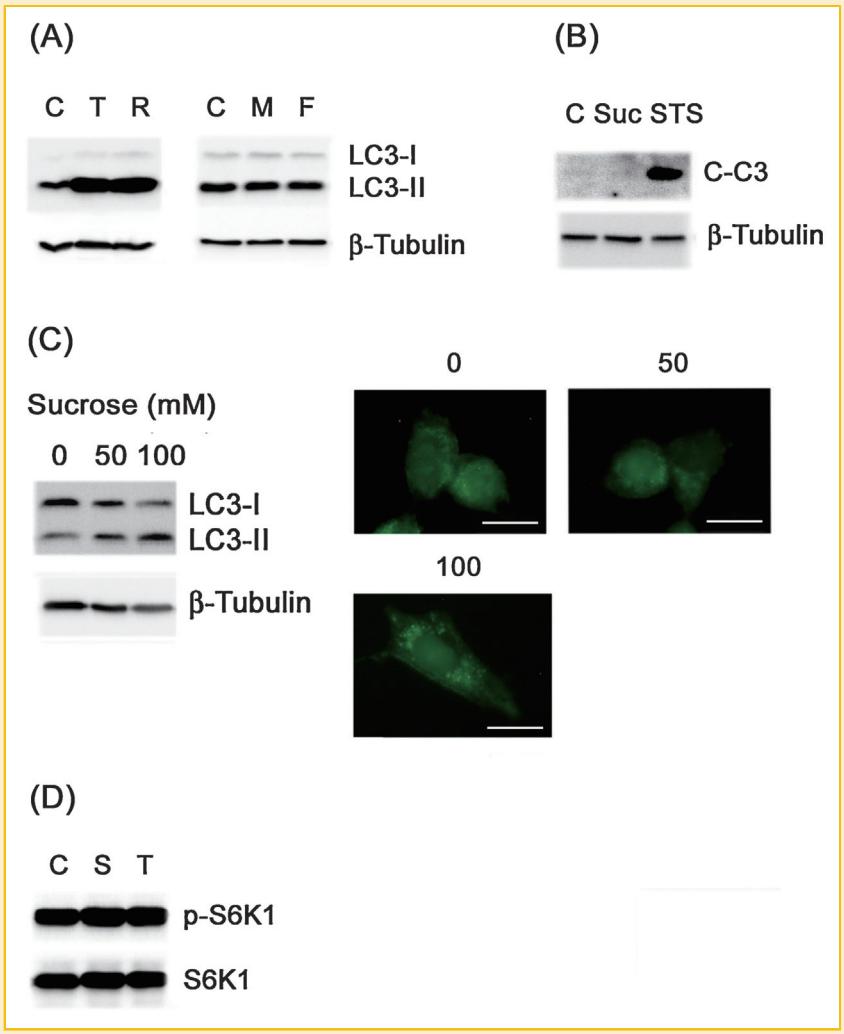

Fig. 2. Effects of saccharides on autophagy related protein expression. (A) MEFs were incubated with 50 mM monosaccharide (fructose, F), disaccharide (maltose, M and trehalose, T) or trisaccharide (raffinose, R) for 24 h and LC3-II expression was analyzed. C: control. (B) MEFs were incubated with sucrose (Suc, 50 mM) or staurosporine (STS, 30 nM) for 24 h, and cleaved caspase-3 (C-C3) expression was analyzed by anti-cleaved caspase-3 antibody. C: control. (C) SH SY5Y cells were incubated with the indicated concentration of sucrose for 24 h and LC3-II expression was analyzed. LC3-II levels in 50 mM and 100 mM sucrose treated cells were 187 ± 28% and 299 ± 49% of control, respectively. SH SY5Y cells transfected with GFP-LC3 were incubated with the indicated concentration of sucrose for 24 h, and the GFP signals were analyzed by fluorescence microscopy. Bar: 20 μm. (D) MEFs were incubated with 50 mM sucrose (S) or trehalose (T) for 24 h and S6K1 phosphorylated at Thr389 (p-S6K1) and total S6K1 were analyzed. C: control.

polyacrylamide gels. The resolved proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane that was incubated with the primary antibody, followed by incubation with the HRP-linked secondary antibody. The blots were developed using EzWestLumi plus (ATTO Corporation, Tokyo, Japan). β-tubulin was used as a loading control.

[^{14}C]-SUCROSE UPTAKE AND RELEASE ASSAY

MEFs were seeded in 12-well plates and incubated for the indicated times with 0.2 μCi/ml [^{14}C]-sucrose in the presence of unlabeled sucrose. Because hyperosmolar solutions are thought to depress linearity of sucrose uptake [Sasaki et al., 1987], appropriate concentrations of NaCl were added to the sucrose solution to maintain osmolality. For the release assays, MEFs were radiolabeled for 24 h as described above, washed with ice-cold serum-free DMEM, and reincubated with sucrose-free incubation medium. At intervals (15–30 min) over the next 3 h, the medium was collected and replaced with fresh medium. At the end of the experiment, cells were washed with ice-cold serum-free DMEM and lysed overnight using 0.05% Triton-X 100/0.1 M NaOH. Radioactivity was measured using a liquid scintillation counter. Protein concentrations were determined using the protein-assay kit.

DIGITONIN TREATMENT

An appropriate concentration of digitonin, allowing the selective extraction of the soluble cellular cytosolic contents without a

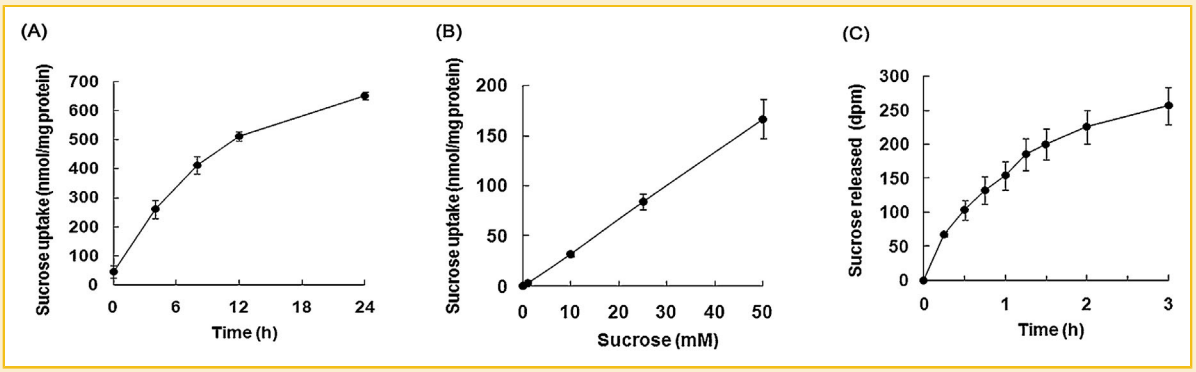

Fig. 3. [^{14}C] -sucrose uptake into cells. (A) MEFs were treated with 50 mM [^{14}C]-sucrose for indicated times and cellular radioactivity was counted. (B) MEFs were incubated with the indicated concentration of [^{14}C]-sucrose for 24 h and radioactivity of cells was counted. (C) MEFs were incubated with 50 mM [^{14}C]-sucrose for 24 h, washed, and incubated with sucrose-free medium. Every 15–60 min the medium was collected and replaced with fresh one. Radioactivity of the medium was counted and the cumulative value plotted at each time point.

significant disruption of lysosomes, was determined using methods described previously [Eboue et al., 2003; Garcia-Garcia et al., 2005] with minor modifications. After MEFs were collected and suspended with DMEM, aliquots of cells were transferred to 1.5 mL tubes and centrifuged at 4 °C for 5 min. The cell pellet was resuspended in digitonin dissolved in buffer solution (0.25 M sucrose, 20 mM HEPES, 2 mM potassium phosphate, 0.24 mM EGTA and 10 mM $MgCl_2$, pH 7.2) at 4 °C for 10 min and centrifuged for 5 min. The supernatants were used to determine lactate dehydrogenase (LDH) activity, a cytoplasmic enzyme, and cathepsin B, a lysosomal enzyme (see below). At a digitonin concentration of 0.005% (w/v), LDH and cathepsin B activities were approximately 95% and 15% of maximal, respectively. Therefore, for further testing this digitonin concentration was used. After incubation of MEFs with 50 mM [^{14}C]-sucrose for 3 h, cells were washed and centrifuged. The cell pellet was incubated in 0.005% (w/v) digitonin solution for 10 min and centrifuged. Using a liquid scintillation counter the radioactivity of the supernatant (cytosol) and the precipitate (vesicular compartments) was counted.

ENZYME ACTIVITY ASSAY

LDH was measured using the oxidation reaction of β-NADH. Aliquots of digitonin soluble fractions were incubated with the reaction mixture (0.1 mM sodium pyruvate, 0.1 mM NADH and 0.1 M sodium phosphate buffer, pH 7.3). The initial rate of absorbance decrease in NADH was quantified by measuring the absorbance for 2 min at a wavelength of 340 nm using a spectrophotometer (SmartSpec Plus, Bio-Rad, Hercules, CA). Cathepsin B was assayed with the substrate BANA, the hydrolysis of which results in fluorescent 2-naphthylamine [Eboue et al., 2003; Garcia-Garcia et al., 2005]. The reaction was carried out at 37 °C for 1 h and was stopped by adding methanol. The amount of 2-naphthylamine at the end of the reaction was determined by Multi-Detection Microplate Reader PowerscanHT (DS Pharma Biomedical, Osaka, Japan) with a 360 nm excitation filter and a 460 nm emission filter. The maximal activity of the enzyme was determined by treating the cells with 1% Triton-X 100. The data are expressed as a percentage of the maximal release.

STAINING OF CELLS WITH LUCIFER YELLOW AND FM-64

MEFs were seeded on cover slips or glass bottom dishes, and incubated for 24 h with Lucifer yellow (0.1 mg/mL) or FM4–64 (1 μg/mL). LysoTracker Red (50 nM) was loaded for 30 min. After washing with PBS, cells were examined using a fluorescence microscope (ECLIPSE E600, Nikon, Tokyo, Japan) or a confocal fluorescence microscope (FV1000-IX81-S, Olympus, Tokyo, Japan).

TRANSMISSION ELECTRON MICROSCOPY

For transmission electron microscopy (TEM), MEFs were fixed in phosphate buffered 2% glutaraldehyde solution, and subsequently post-fixed in 2% osmium tetroxide for 3 h in the ice bath. Then, the specimens were dehydrated in graded ethanol solutions and embedded in epoxy resin. Ultrathin sections were obtained by the ultramicrotome followed by staining with uranyl acetate for 10 min and with a lead staining solution for 5 min. The specimens were then analyzed using a TEM (JEM-1200 EX, JEOL, Japan).

CELL VIABILITY ASSAY

Cells were seeded in 96-well plates and incubated for 24 h with 50 mM sucrose. The precipitated crystals of formazan, formed after 2 h incubation with MTT (0.5 mg/mL), were dissolved by adding DMSO. The absorbance values of the final solution were determined at 570 nm, with 630 nm as the reference wavelength. All MTT assays were performed in triplicates. The data (the absorbance at 570 nm minus the absorbance at 630 nm) were expressed as percentage of the untreated cells.

STATISTICAL ANALYSIS

Data are presented as the mean ± standard error of the mean (SEM) using at least three independent experiments. Statistical analyses were performed with Student's *t*-test or one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

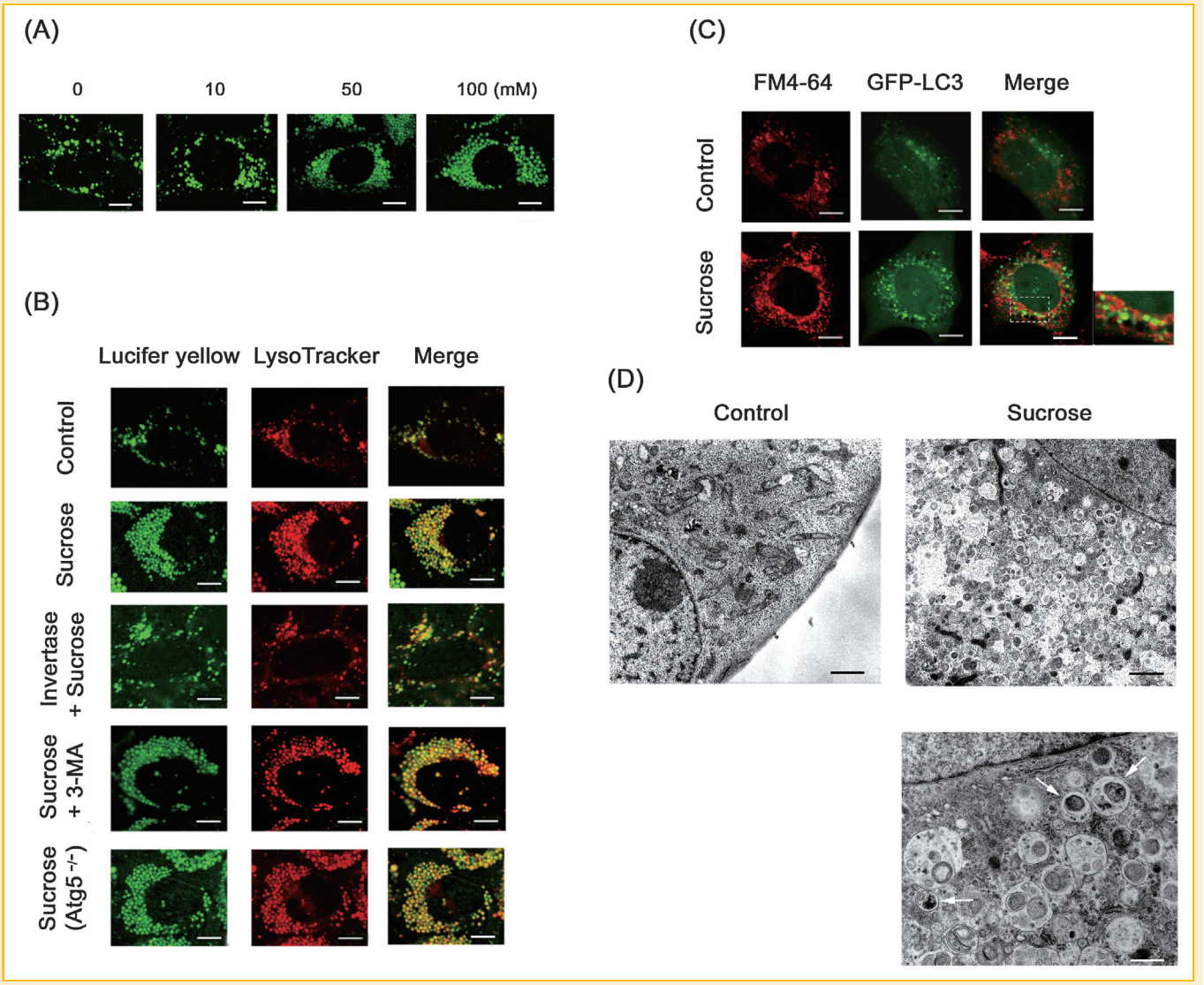

Fig. 4. Vesicle accumulation induced by sucrose. (A) MEFs were incubated with the indicated concentration of sucrose for 24 h in the presence of Lucifer yellow, and analyzed by confocal fluorescence microscopy. Bar: 10 μm. (B) MEFs were pretreated with or without invertase or 3-MA and incubated with 50 mM sucrose for 24 h in the presence of Lucifer yellow. LysoTracker Red was loaded for the last 30 min. Atg5$^{-/-}$ cells were incubated with 50 mM sucrose for 24 h. Fluorescence signals were analyzed by confocal fluorescence microscopy. Bar: 10 μm. (C) MEFs transfected with GFP-LC3 were incubated with 50 mM sucrose in the presence of FM4–64 for 24 h, and analyzed by confocal fluorescence microscopy. Bar; 10 μm. (D) Electron micrographs of MEFs treated with 50 mM sucrose for 24 h. Bar; 2 μm (control and upper panel of sucrose) and 0.5 μm (bottom panel of sucrose). Arrows indicate autophagic vesicles.

RESULTS

SUCROSE-INDUCED AUTOPHAGY

To examine whether sucrose induces autophagy, the effects of sucrose on the expression of microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy, were analyzed by western blotting. Cytosolic LC3 (LC3-I, 16 kDa) is converted to LC3-II (14 kDa), which associates with autophagosome membranes, thereby, serving as a marker of autophagy [Kabeya et al., 2000]. Incubation for 24 h of wild-type mouse embryonic fibroblasts (MEFs) with sucrose at concentrations over 10 mM resulted in the upregulation of LC3-II levels (Fig. 1A). The increase in LC3-II levels in cells incubated with 50 mM sucrose was significant at 24 h (Fig. 1B). When 3-methyladenine (3-MA), an inhibitor of autophagy was included with the sucrose, the effect on LC3-II was suppressed (Fig. 1C). To confirm that the LC3-II accumulation was not caused by blocking lysosomal degradation, LC3-II levels were examined following incubation with the lysosomal protease inhibitors E64d and pepstatin A. As shown in Fig. 1D, sucrose treatment augmented the expression of LC3-II in the presence of these inhibitors, indicating that autophagic flux was enhanced by sucrose. Furthermore, GFP-LC3-containing dot structures (autophagosomes) increased after sucrose treatment in a concentration-dependent manner (Fig. 1E).

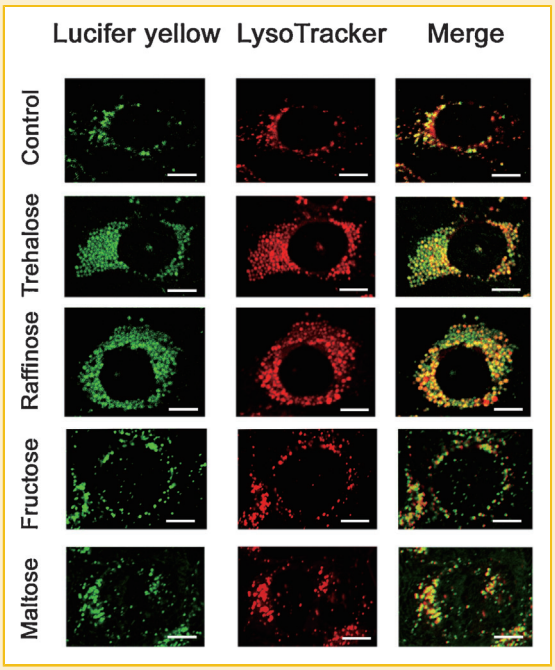

Fig. 5. Vesicle accumulation induced by various saccharides. MEFs were incubated with 50 mM trehalose, raffinose, fructose, or maltose for 24 h in the presence of Lucifer yellow. LysoTracker Red was loaded for the last 30 min. Fluorescence signals were analyzed by confocal fluorescence microscopy. Bar: 10 μm.

We also examined the effects of several saccharides at 50 mM on LC3-II levels and found that the di- and trisaccharides trehalose and raffinose, respectively, increased LC3-II expression, but the monosaccharide fructose and a disaccharide maltose did not (Fig. 2A). Cell viability following treatment with sucrose for 24 h was 104.9% ± 9.0% of control. An increase in the cleaved (active) caspase-3 (19 kDa) was not observed in sucrose-treated cells, although it was apparent in staurosporine-treated cells (Fig. 2B). Incubation of human neuroblastoma SH SY5Y cells with 50 mM and 100 mM sucrose for 24 h increased LC3-II levels, and GFP-LC3 dots were readily observable at 100 mM (Fig. 2C). These results suggested that sucrose induces autophagy, without osmotic or toxic effects.

Since autophagy is induced by inhibiting the target of rapamycin (TOR) [Noda and Ohsumi, 1998; Meijer and Codogno, 2004], which is a downstream target of Akt, we examined the effect of sucrose on TOR activity. Mammalian TOR (mTOR) kinase activity can be determined by measuring phosphorylation of its substrate ribosomal protein S6 kinase 1 (S6K1) at Thr389. As shown in Fig. 2D, phosphorylation of S6K1 in MEFs was not affected in response to sucrose or trehalose. Therefore, sucrose-induced autophagy appeared to be mTOR independent. Autophagy activity is also shown to be linked to ER stress signaling pathways [Ogata et al., 2006], but the level of an ER stress marker (Grp78) was not affected by the sucrose treatment (data not shown).

SUCROSE UPTAKE AND SUCROSE-INDUCED MORPHOLOGICAL CHANGES

It has been shown that various types of cells internalize sucrose and this induces vacuole formation [Cohn and Ehrenreich, 1969; Nyberg and Dingle, 1970; Wagner et al., 1971; DeCourcy and Storrie, 1991; Karageorgos et al., 1997]. To confirm that these events occur under our experimental conditions also, we first examined sucrose uptake using [^{14}C]-sucrose. Radioactivity incorporated into MEFs was increased for 24 h at 37 °C (Fig. 3A) and was proportional to the external sucrose concentrations in a range of 1 mM to 50 mM (Fig. 3B). In contrast, sucrose uptake for 3 h at 4 °C was approximately 10% of that at 37 °C. We also verified the intracellular location of sucrose using digitonin, as described in the Materials and Methods section, and found that 63.3% ± 6.0% of the total radioactivity was present in vesicular compartments. When cells were reincubated with sucrose-free medium after incubation with [^{14}C]-sucrose for 24 h, radioactivity was released into the medium (Fig. 3C) as was previously shown [Besterman et al., 1981; Sasaki et al., 1987]. The cellular radioactivity following 3 h of reincubation was 82.3% ± 2.1% when compared to that at 0 h.

To examine the morphological changes in sucrose-treated MEFs, cells were incubated with the fluorescence dye Lucifer yellow. It is known that this dye is similar in size to sucrose and is taken up via pinocytosis. As shown in Fig. 4A, small vesicles were randomly distributed in the control cells. However, the cytoplasm of sucrose-treated cells contained various sized vesicles, which increased in a concentration-dependent manner. Labeling the cells with Lucifer yellow and LysoTracker Red, a fluorescent dye for the acidic organelles, indicated that a considerable part of the sucrose-induced vesicles were acidic compartments (Fig. 4B). Therefore, it appears that sucrose is internalized through pinocytosis and accumulates in endosomes/lysosomes. When cells were preincubated with the sucrose-degrading enzyme invertase in order to allow its internalization, the vesicles number and size decreased significantly (Fig. 4B). Autophagy inhibition by 3-MA or by knockdown of Atg5 gene did not affect vesicle accumulation (Fig 4B), suggesting that these vesicles were not induced by autophagy. Therefore, it appears that sucrose is internalized through pinocytosis and accumulates in endosomes/lysosomes vesicles.

Furthermore, GFP-LC3-transfected MEFs were incubated with the red fluorescent dye FM4–64, which binds to plasma membrane and to be internalized by endocytosis. After the treatment of cells with 50 mM sucrose for 24 h, FM4–64-labeled vesicles were observed throughout the cytoplasm, and GFP-LC3 dots were partially colocalized with them (Fig. 4C). Electron microscope analysis demonstrated that sucrose–treated cells contained vesicles of various sizes, some of which appeared to be autophagic vesicles, autophagosomes and autolysosomes (Fig. 4D).

THE RELATIONSHIP BETWEEN VESICLE ACCUMULATION AND AUTOPHAGY

When MEFs were treated with saccharides, which were used to examine the effects on LC3-II expression (Fig. 2A), vesicle accumulation was induced by trehalose and raffinose, but not by fructose and maltose (Fig. 5). These results indicated that vesicle accumulation was associated with autophagy. We sought to verify this interpretation by means of cells pretreatment with invertase. It was found that LC3-II level and autophagosome content increase while vesicle accumulation following sucrose treatment was inhibited (Fig. 6A). Incubation of cells with sucrose for 24 h followed

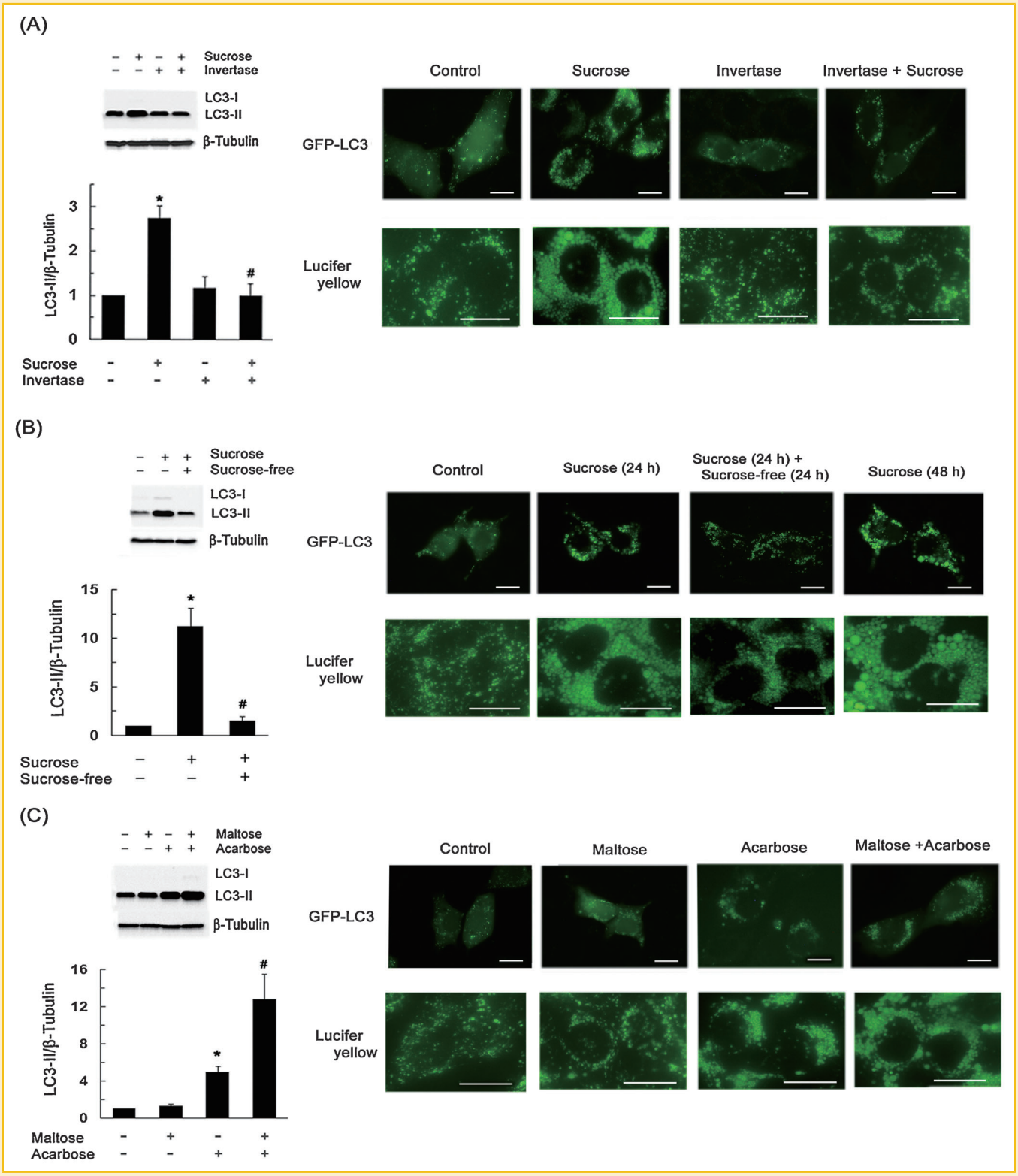

Fig. 6. The correlation between LC3-II level, autophagosome content and vesicle accumulation. (A) MEFs were pretreated with invertase and incubated with 50 mM sucrose for 24 h, and LC3-II expression was analyzed. *$P < 0.05$ versus without treatment; $^{\#}P < 0.05$ versus sucrose alone. MEFs transfected with GFP-LC3 were pretreated and incubated as described above and analyzed by fluorescence microscopy. MEFs were pretreated and incubated in the presence of Lucifer yellow as described above and analyzed by fluorescence microscopy. (B) MEFs were incubated with 50 mM sucrose for 24 h and then a sucrose-free medium for 24 h, and LC3-II expression was analyzed. *$P < 0.05$ versus without treatment; $^{\#}P < 0.05$ versus sucrose alone. MEFs transfected with GFP-LC3 were incubated as described above and analyzed by fluorescence microscopy. MEFs were incubated in the presence of Lucifer yellow as described above and analyzed by fluorescence microscopy. (C) MEFs were incubated with 50 mM sucrose with or without 7.7 mM acarbose for 24 h and LC3-II expression was analyzed. *$P < 0.05$ versus without treatment; $^{\#}P < 0.05$ versus maltose alone. MEFs transfected with GFP-LC3 were incubated as described above and analyzed by fluorescence microscopy. MEFs were treated in the presence of Lucifer yellow as described above and analyzed by fluorescence microscopy. Bar: 20 μm.

by incubation in a sucrose-free medium, which decreased [^{14}C]-sucrose content in cells as described above, downregulated theLC3-II level and autophagosome formation, while reducing the vesicle size (Fig. 6B). Furthermore, we used a α-glucosidase inhibitor acarbose that suppressed maltose hydrolysis. As shown in Fig. 6C, maltose plus acarbose enhanced LC3-II and autophagosome accumulation, and vesicles number and size. Acarbose alone upregulated these events, probably because acarbose is a pseudotetrasaccharide. Taken together, swollen vesicle accumulation induced by non-hydrolysable saccharides appears to be associated with autophagy.

DISCUSSION

In this study, we showed that sucrose induces autophagy in a concentration- and time-dependent manner. It has been demonstrated that sucrose, unlike trehalose, did not reduce mutant huntingtin aggregates and toxicity by autophagy, although the effect on LC3-II levels was not described [Sarkar et al., 2007]. Trehalose protects the integrity of cells against environmental stresses, due to its physical and chemical properties [Crowe et al., 1984; Argüelles, 2000], and regulates signaling pathways, which are different from those of sucrose (Helip-Wooley and Thoene, 2004; Minutoli et al., 2007). Therefore, trehalose may have effects other than autophagy induction on huntingtin aggregates. Furthermore, we examined whether other sugars enhanced LC3-II expression. Raffinose as well as trehalose, upregulated LC3-II expression, while fructose and maltose had no effect on it. These results suggest that non-hydrolyzed saccharides induce autophagy.

Sucrose has been shown to be taken up into cells and to induce vacuolation, although it does not have any toxic effects [Cohn and Ehrenreich, 1969; Nyberg and Dingle, 1970; Wagner et al., 1971; DeCourcy and Storrie, 1991; Karageorgos et al., 1997]. Therefore, we examined sucrose uptake and sucrose-induced morphological changes using [^{14}C]-sucrose and fluorescent tracers of endocytosis. We found that sucrose was internalized by pinocytosis and induced formation of numerous vesicles of various sizes. These vesicles appeared to be sucrose-containing endosomes/lysosomes, and to be fused with autophagic vesicles, since they were colocalized with LysoTracker and/or GFP-LC3 dot structures. It has been suggested that endocytosis is coupled to autophagy (Seglen and Bohley, 1992; Lamb et al., 2013) and that autophagosomes fuse with the endosomes [Berg et al., 1998; Fader et al., 2008]. On the other hand, large vesicles were probably caused by the swelling and fusion of endocytic vesicles as previously demonstrated [Wagner et al., 1971; DeCourcy and Storrie, 1991]. Therefore, it appears that sucrose is taken up into cells and that it promotes formation of vesicles, endosomes/lysosomes, which are fused with each other and autophagic vesicles. It should be noted that these vesicles are packed within the cytoplasm, because sucrose was not hydrolyzed in the lysosomes.

To verify that autophagy was associated with the accumulation of vesicles, we determined the relationship between these events. Cells exposed to several saccharides with Lucifer yellow showed enhanced vesicle formation and elevated LC3-II levels in the cells treated with trehalose and raffinose, but not with other saccharides. The correlation between the amount of vesicles and induction of autophagy was confirmed by the treatment of cells with invertase, sucrose deprivation or maltose plus acarbose. The methods to induce autophagy inhibition did not affect vesicle formation. It has been reported that cytoplasmic vacuoles accumulation, which was caused by hyperstimulation of macropinocytosis with nerve growth factor in TrkA receptor expressed cells induced autophagy, while inhibition of autophagy did not block vacuole formation [Li et al., 2010]. Taken together, these data suggest that the accumulation of a large amount of vesicles results in induction of autophagy.

Although the mechanism of sucrose-induced autophagy is unknown, the present study proposes that autophagy is induced to attenuate the crowding effects in cytoplasm, which is occupied by sucrose-containing vesicles. It has been demonstrated that macromolecular crowding affects the rate of biochemical reactions [Minton, 2001; Miyoshi and Sugimoto, 2008]. This tends to increase reaction rates by increasing enzyme concentrations and tends to decrease reaction rates by reducing the metabolites diffusion coefficient [Vazquez, 2010]. Therefore, the molecular crowding may affect the functions of autophagy-related proteins. Hyperosmotic stress, which leads to macromolecular crowding, was also shown to induce autophagy [Han et al., 2010; Nunes et al., 2013]. It is probable that similar events occur in some types of lysosomal storage disease. Extended cytoplasmic vacuolation and enhanced autophagy were observed in diseases, such as GM1-gangliosidosis and mucolipidosis [Itoh et al., 2001; Takamura et al., 2008; Schweizer et al., 2013; Lieberman et al., 2012]. Further studies would be required to elucidate the relationship between vesicle accumulation and autophagy.

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