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Alkaline Stress-Induced Autophagy is Mediated by mTORC1 Inactivation

Jinkyu Suk,[1] Sang Su Kwak,[1] Jae Ho Lee,[1] Ji Hye Choi,[1] Sang-Hee Lee,[1] Dong Hwan Lee,[2] Boohyeong Byun,[3] Geon-Hyoung Lee,[4] and Cheol O. Joe[1*]

[1]*Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea*

[2]*Department of Pediatrics, College of Medicine, Soonchunhyang University, Seoul 336-745, South Korea*

[3]*Department of Oriental Medicine, Daegu Haany University, Daegu 705-060, South Korea*

[4]*Department of Biology, Kunsan National University, Chonbuk 573-701, South Korea*

ABSTRACT

The activation of autophagic pathway by alkaline stress was investigated. Various types of mammalian cells were subjected to alkaline stress by incubation in bicarbonate buffered media in humidified air containing atmospheric 0.04% CO_2. The induction of autophagy following alkaline stress was evaluated by assessing the conversion of cytosolic LC3-I into lipidated LC3-II, the accumulation of autophagosomes, and the formation of autolysosomes. Colocalization of GFP-LC3 with endolysosomal marker in HeLa GFP-LC3 cells undergoing autophagic process by alkaline stress further demonstrates that autophagosomes triggered by alkaline stress matures into autolysosomes for the lysosome dependent degradation. We found that the inactivation of mTORC1 is important for the pathway leading to the induction of autophagy by alkaline stress since the expression of RhebQ64L, a constitutive activator of mTORC1, downregulates the induction of autophagy after alkaline stress in transfected human 293T cells. These results imply that activation of autophagic pathway following the inactivation of mTORC1 is important cellular events governing alkaline stress-induced cytotoxicity and clinical symptoms associated with alkalosis. J. Cell. Biochem. 112: 2566–2573, 2011.

KEY WORDS: pH; AUTOPHAGY; ALKALINE STRESS; mTOR; LC3

The induction of intracellular alkalization (alkaline stress) is not a rare event. Alkalosis, a process which raises the cellular pH, is associated with various clinical conditions. Respiratory alkalosis is believed to impair the central and peripheral nervous system [Galla, 2000]. Hyperammonia is commonly seen in patients with liver diseases or inherited disorder of urea cycle enzymes. When the liver fails to remove ammonia, a rapid increase in the concentration of ammonia triggers alkaline stress in all types of cells including astrocytes and neurons [Marcaggi and Coles, 2001; Rose et al., 2005]. Cytoplasmic pH is important for a diverse cellular functions including metabolic enzyme activity, cytoskeleton assembly, ion conductivities, and cytokine and mediator release [Regula et al., 1981; Madshus, 1988; Gillies et al., 1990; Vairo et al., 1992; Xu and Fidler, 2000]. There are studies demonstrating protein unfolding [Bai et al., 1998] and changes in enzymatic activities [Murachi and Yamazaki, 1970] in cells under alkaline conditions. Increased levels of mitochondrial transmembrane potential followed by mitochondrial damages in murine fibroblasts grown under alkaline conditions have suggested that mitochondria are primary target organelles of injury by alkaline stress [Majima et al., 1998].

This study first reports alkaline stress as one of agents that induce autophagy (macroautophagy). Autophagy is a process of remodeling endosomal membrane structure that engulfs lysosome-dependent degradation of long-lived cytosolic proteins, large protein aggregates, and damaged organelles [Eskelinen, 2005; Mizushima et al., 2008]. It is reasonable to assume that this remodeling process should be regulated in response to environmental stress in order to maintain healthy cytoplasmic constituents. Recently, Eng et al. [2010] have presented a strong supporting evidence for a possible link between alkaline stress and autophagy induction. Cancer cells are heavily

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*Correspondence to: Cheol O. Joe, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea. E-mail: cojoe@kaist.ac.kr

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dependent on glycolysis for energy production and produce ammonia derived from the deamination of glutamine. Ammonia which triggers the raise of intracellular pH has been shown to be a diffusible inducer of autophagy in cultured human cells [Eng et al., 2010].

Studies have documented that mTOR, an evolutionary conserved Ser/Thr protein kinase, is a central controller of cell cycling, cell size, cell shape, and cell proliferation. Structurally, mTOR forms two complexes in cells in which mTOR is associated with either Raptor (mTORC1) or Rictor (mTORC2) [Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2005; Wullschleger et al., 2006]. While mTORC2 is a rapamycin-resistant protein kinase that phosphorylates and activates Akt, mTORC1 is activated downstream of Akt and regulates various cellular functions including protein synthesis and cell size [Fingar and Blenis, 2004; Laplante and Sabatini, 2009]. The activity of mTORC1 as a protein kinase is regulated by Rheb, a Ras-related GTPase protein, which is in turn negatively regulated by Tuberous Sclerosis Complex, TSC1/2. Akt has been known to activate Rheb through the inactive phosphorylation of TSC2. Upon upstream inputs including growth factors, GTP bound form of Rheb activates mTORC1. Rheb-GTP binds directly to the amino-terminal lobe of mTOR catalytic domain to regulate its kinase activity. RhebQ64L is a mutant form of Rheb which is mostly bound to GTP, thereby constitutively activating mTORC1 [Long et al., 2005]. In addition, TSC2 is activated by AMPK which senses the cellular energy state and is activated by a high ratio of AMP to ATP. Under the state of cellular energy depletion, AMPK phosphorylates TSC2 at active site, leading to the inhibition of mTORC1 function [Wullschleger et al., 2006]. Activation of mTORC1 has been known to coordinate protein synthesis as well as cell growth. The mTORC1 phosphorylates S6K, which directly phosphorylates 40S ribosomal protein S6, and upregulates the capacity of translational machinery [Balgi et al., 2009]. The mTORC1 also phosphorylates 4E-BP, facilitating the dissociation of 4E-BP from eIF4E, thereby promoting cap-dependent translation initiation [Fingar et al., 2002].

Autophagy is regulated either by mTORC1-dependent or independent pathway. Induction of autophagy by deficiencies of growth factors or nutrients is mediated by mTORC1, which negatively regulates autophagic pathway [Fingar and Blenis, 2004; Jung et al., 2010; Wullschleger et al., 2006]. Although the exact mechanism is unknown, autophagy has been also described to be negatively regulated by a cyclical mTOR-independent pathway. In this pathway, Ca^{2+} channel antagonists increase intracytosolic Ca^{2+} activating Ca^{2+}-dependent cysteine proteases called calpains, which cleaves and activates Gs_{α} required for the catalytic stimulation of adenylyl cyclase. Elevated cAMP level by adenylyl cyclase in turn results in the production of IP_3 and consequent release of Ca^{2+} from the endoplasmic reticulum forming a cyclical signaling pathway. Activation of this cyclical pathway has been found to inhibit autophagy [Hoyer-Hansen et al., 2007; Williams et al., 2008; Sarkar et al., 2009] in mammalian cell and animal models [Gordon et al., 1993].

Here, we report that alkaline stress induces autophagy in cultured mammalian cells and propose the important role of mTORC1 on the induction of autophagy in cells under alkaline conditions.

MATERIALS AND METHODS

CELL CULTURE

Cell lines except CHO (Chinese Hamster Ovary) were cultured in bicarbonate-buffered DMEM containing 3.7 g/L sodium bicarbonate ($NaHCO_3$) supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY). CHO cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, CA) supplemented with 10% dialyzed fetal bovine serum (dFBS, Invitrogen) and HT supplement (Gibco). Cells were normally maintained at 37°C in an incubator with a normal flow of 5% (v/v) CO_2. Cells were grown in air with 0.04% atmospheric CO_2 at 37°C to evaluate the effect of alkaline stress. pHs of growth media were measured using a calibrated pH meter.

PLASMIDS AND ANTIBODIES

cDNA encoding mouse Rheb was cloned into EcoRI and BamHI sites of expression vector pFlag-CMV2 (Sigma, St. Louis, MO) to produce pFlag-Rheb vector using PCR primers 5′-CGGAATTCAATGCCTCAGTCCAA-3′ and 5′-CGGGATCCTCACATCACCGAGC-3′. The mutant construct containing Q64L substitution was generated by using two-step PCR-based targeted mutagenesis. RhebQ64L was cloned into EcoRI and BamHI sites of expression vector pFlag-CMV2 to produce pFlag-RhebQ64L vector. Specific primers used to amplify the substituted fragment were 5′-AGACACAGCGGGGCTGGATGAAT-3′ and 5′-ATTCATCCAGCCCCGCTGTGTCT-3′. Antibody against tubulin was purchased from Sigma. Anti-LC3 rabbit polyclonal antibody was from Novus Biologicals (Littleton, CO). Anti-GFP (Green Fluorescent Protein) antibody was supplied by Santa Cruz (Santa Cruz, CA). Rabbit monoclonal antibody specific for phosphorylated S6K at Thr 389 was from Cell Signaling (Boston, MA). Rabbit antibodies against Akt and phosphorylated Akt at Thr 308 or Ser 473 were also from Cell Signaling.

ESTABLISHMENT OF HELA GFP-LC3 CELLS

293T cells were cotransfected with retroviral pRevExTO2 expression vector containing cDNA encoding N-terminal GFP fusion of LC3 (GFP-LC3) and pGP expression vector for MuLV Gag-Pol (Takara, Otsu, Shiga, Japan) together with pVpack-VSV-G expression vector encoding VSV-G protein (Stratagene, Santa Clara, CA). Viral particles were collected after 48 h, filtered, and used to infect HeLa cells. Infected HeLa cells were cultured in the presence of 8 μg/ml Polybrene (Sigma) to select cells expressing GFP-LC3. Transduced HeLa GFP-LC3 cells were then selected in DMEM containing 3 μg/ml puromycin to establish stable subclones.

QUANTIFICATION OF AUTOPHAGY

The percentage of GFP-LC3 positive HeLa GFP-LC3 cells with GFP-LC3 punctate dots was determined as described [Fimia et al., 2007]. Briefly, HeLa GFP-LC3 cells were grown on coverslips, fixed with 3.7% formaldehyde in PBS, washed three times, and mounted for examination by fluorescence microscopy.

COLOCALIZATION OF LYSOTRACKER RED® WITH GFP-LC3

HeLa GFP-LC3 cells were incubated under alkaline stress ($7.4 < pH < 8.2$) for 2 h and washed twice with PBS. LysoTracker

Red® (LTR) DND-99 labeling solution (Invitrogen, Carlsbad, CA) was applied to HeLa GFP-LC3 cells for 1 min at room temperature as specified by the manufacturer's procedures. The labeling solution was removed by washing cells with PBS. Fixing procedure with 3.7% formaldehyde was omitted. Colocalization of GFP-LC3 and LTR in cells after alkaline stress was then imaged under a confocal laser scanning microscope (Carl-Zeiss, Thornwood, NY).

ELECTRON MICROSCOPY

HeLa cells were fixed with 1% glutaraldehyde in PBS for 2 h at 4°C, washed five times with 0.1 M cacodylate buffer containing 0.1% $CaCl_2$ at 4°C, and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) containing 0.1% $CaCl_2$ for 1 h at 4°C. After dehydration and embedding in Embed-812 (EMS, Hatfield, PA), ultrathin sections were contrasted with uranyl acetate and lead citrate and photographed in H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

FLOW CYTOMETRY

Cell lines were grown in an incubator with a flow of 5% (v/v) CO_2 or incubated in air with 0.04% atmospheric CO_2 for 10 h ($7.4 < pH < 8.8$) to evaluate the effect of alkaline stress on cell size. Cells were fixed with ice-cold 70% ethanol and incubated on ice for 30 min. Fixed cells were then treated with RNaseI and stained with 10 μg/ml propidium iodide (PI). DNA contents of cells at G1 phase were measured by flow cytometry to determine Forward Scatter Height (FSC-H) parameter as a measure of relative cell size.

RESULTS

ALKALINE STRESS INDUCES AUTOPHAGY IN CULTURED MAMMALIAN CELLS

The induction of autophagy by alkaline stress was ascertained by ultrastructure analysis showing the accumulation of autophagosomes in HeLa cells grown under alkaline stress ($7.4 < pH < 8.2$) for 2 h (Fig. 1A). Throughout this study alkaline stress was induced by changing the ambient carbon dioxide concentration (pCO_2) in an incubator from normal 5% to atmospheric 0.04%. The pHs of growth media were measured at stated period of time after incubation of cells in air (0.04% CO_2) at 37°C. In response to alkaline stress, LC3-I was converted into LC3-II, a hallmark of autophagy [Levine and Kroemer, 2008], in several human and murine cell lines (Fig. 1B). The accumulation of GFP-LC3 in autophagosomes under alkaline stress for 2 h ($7.4 < pH < 8.2$) was imaged by confocal microscopy and quantified in established HeLa GFP-LC3 cells stably expressing GFP-LC3 (Fig. 1C). Alkaline stress increased the frequency of cells with GFP-LC3 punctate dots by approximately sixfold (Fig. 1D).

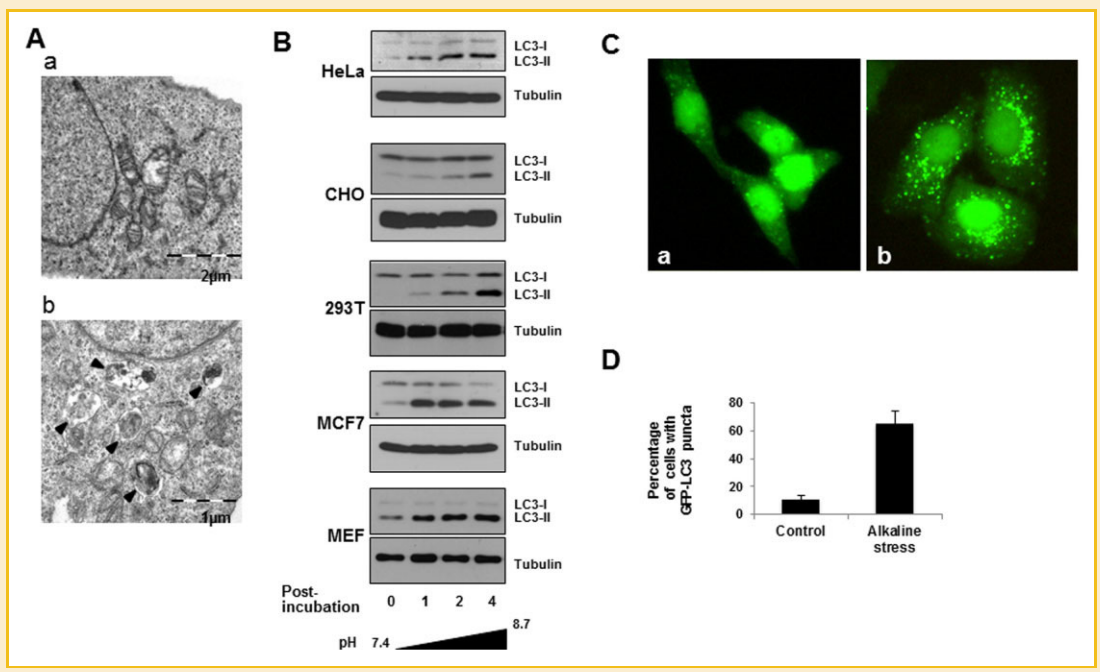

Fig. 1. Alkaline stress induces autophagy in cultured mammalian cells. A: Representative electron micrographs of autophagic HeLa cells grown under alkaline stress ($7.4 < pH < 8.2$) for 2 h. Alkaline stress was given by changing the ambient carbon dioxide concentration (pCO_2) from normal 5% to atmospheric 0.04%. The pHs of growth media were measured by pH meter after alkaline stress was given. Control HeLa cells were maintained in an incubator with a normal flow of humidified 5% CO_2 at 37°C (a). Cells were incubated under alkaline stress ($7.4 < pH < 8.2$) for 2 h. Arrowheads indicate the formation of autophagosomes (b). B: Cells were incubated under normal conditions (pH 7.4, lane 1) or alkaline stress for 1, 2, or 4 h ($7.4 < pH < 8.7$, lanes 2–4). Cells were examined for the conversion of LC3-I into LC3-II by Western Blot analysis using LC3 antibody. C: HeLa GFP-LC3 cells stably expressing GFP-fused LC3 were incubated under alkaline stress ($7.4 < pH < 8.2$) for 2 h. The accumulation of GFP-LC3 in autophagosomes in cells before (a) or after alkaline stress (b) was assessed by fluorescence microscopy. D: Autophagy induction in HeLa GFP-LC3 cells after alkaline stress for 2 h was quantified by counting the number of cells with GFP-LC3 punctate dots. Autophagy induction was quantified as mean ± S.D. of combined results from at least three independent experiments.

MATURATION OF ALKALINE STRESS-INDUCED AUTOPHAGOSOMES INTO AUTOLYSOSOMES

To determine whether alkaline stress-induced autophagosomes can proceed to degradative autolysosomes, we examined autophagic flux during the course of autophagosome maturation in HeLa GFP-LC3 cells after alkaline stress. Autophagic flux was measured by monitoring GFP-LC3 processing and its degradation. When autophagosomes fuse with lysosomes, GFP-LC3 in the inner surface of autophagosomes is to be degraded by lysosomal hydrolases. Since LC3 is degraded faster than the stable GFP moiety, GFP transiently accumulates in the lysosome [Balgi et al., 2009]. Accordingly, the accumulation of GFP moiety was examined as a measure for autophagic flux whereas the conversion of GFP-LC3-I into GFP-LC3-II was monitored as a measure for the induction of autophagosome in HeLa GFP-LC3 cells. Incubation of HeLa GFP-LC3 cells under alkaline stress for 4 h ($7.4 < pH < 8.7$) caused a rapid accumulation of lipidated GFP-LC3-II and GFP, indicating that alkaline stress induces autophagosomes and increases autophagic flux as well (Fig. 2A). Maturation of autophagosomes to autolysosomes in HeLa GFP-LC3 cells under alkaline stress was also evaluated by examining colocalization of GFP with endolysosomal marker LysoTracker Red® (LTR). LTR selectively accumulates in acidic cellular compartment and is frequently used as a fluorescent probes for labeling and tracking acidic organelles such as the lysosome [Freundt et al., 2007]. Almost complete overlapping of fluorescence from GFP (green) with LTR (red) implies that autophagosomes induced by alkaline stress fuse with lysosomes efficiently to form mature autolysosomes (Fig. 2B).

mTORC1 SIGNALING IS IMPORTANT FOR AUTOPHAGY INDUCTION BY ALKALINE STRESS

Inactivation of mTORC1 has been shown to induce autophagy in order to maintain cellular homeostasis by facilitating the turnover of damaged proteins and organelles [Eskelinen, 2005; Mizushima et al., 2008]. Our data in Figure 3 indicate that alkaline stress-induced autophagy is mediated by the inhibition of mTORC1. The involvement of mTORC1 in alkaline stress-induced autophagy was examined in transfected 293T cells overexpressing RhebQ64L, a constitutively active mutant of Rheb. Since Rheb is a positive regulator of mTOR kinase, RhebQ64L functions as an activator of mTORC1 [Jiang and Vogt, 2008]. Expression of RhebQ64L increased mTORC1 activity in transfected cells as assessed by the increased phosphorylation of S6K at Thr 389. However, the induction of autophagy as seen by the conversion of LC3-I into LC3-II was reduced by the expression of RhebQ64L in transfected 293T cells grown under alkaline stress (Fig. 3A). These results imply that alkaline stress-induced autophagy is mediated by mTORC1 inactivation. The mTORC1 kinase activity measured by the phosphorylation of S6K was also inhibited by alkaline stress in other human cells (Fig. 3B). Data in Figure 3C indicate that mTORC1 activity was not affected by acidic stress ($7.0 < pH < 7.4$), suggesting that the inhibition of mTORC1 by pH stress is specific to alkaline conditions (Fig. 3C). When HeLa cells grown under alkaline conditions for 4 h were further incubated in an incubator with a normal flow of 5% CO_2, mTORC1 activity in these cells were almost completely restored (Fig. 3D, lane 5).

ALKALINE STRESS REDUCES CELL SIZE BUT ACTIVATES AKT

Given that alkaline stress inactivates mTORC1 to activate autophagic pathway, we hypothesized that mTORC1 inactivation by alkaline stress should be linked to other cellular events controlled by mTORC1 functions. We evaluated the effect of alkaline stress in controlling cell size in several types of human cells by flow cytometry. After incubation for 10 h under alkaline stress, the relative size of G1-phase HeLa cells was considerably reduced, as seen by the leftward shift of the mean FSC-H histograms. The average size of HeLa cells treated for the same period of time with 100 nM rapamycin, a known inhibitor of mTORC1, was also reduced.

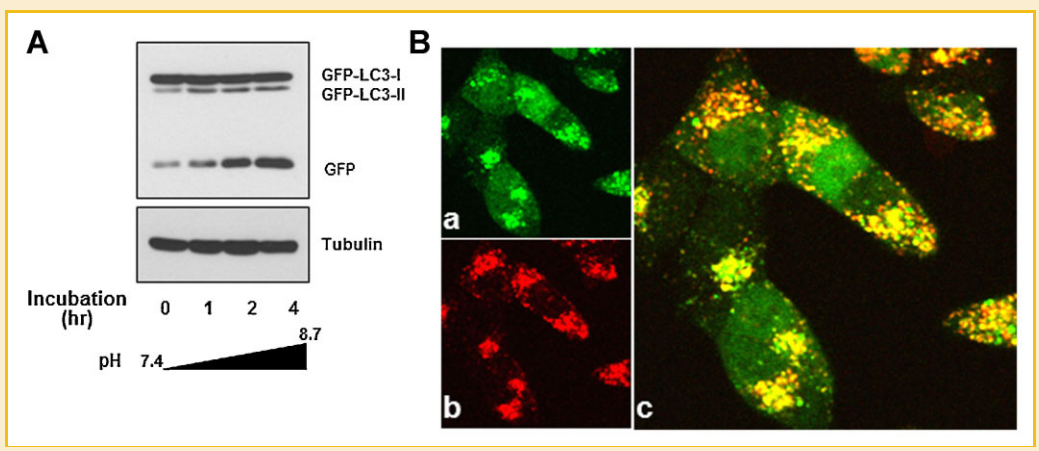

Fig. 2. Maturation of alkaline stress-induced autophagosomes into autolysosomes. A: HeLa GFP-LC3 cells were grown under alkaline stress ($7.4 < pH < 8.7$) for indicated amount of time. The conversion of LC3-I into LC3-II and the accumulation of GFP protein during the course of alkaline stress-induced autophagy were monitored by Western blot analysis. B: HeLa GFP-LC3 cells grown under alkaline stress ($7.4 < pH < 8.2$) for 2 h were examined for the colocalization of GFP-LC3 (green) with the acidic endo/lysosomal marker LTR (LysoTracker Red®) (red). Note that the merged image of cells (c) shows substantial colocalization of GFP staining (a) with LTR staining (b).

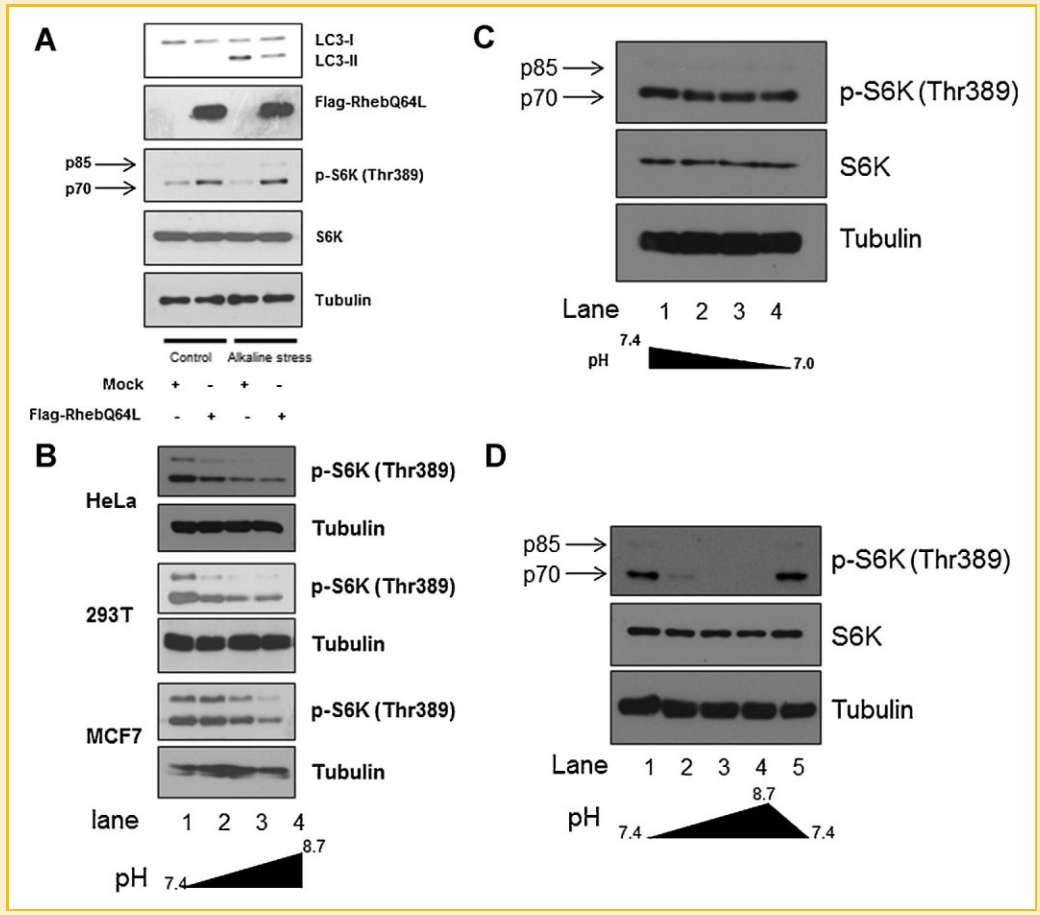

Fig. 3. Important role of mTORC1 signaling on autophagy induction by alkaline stress. A: 293T cells (1×10^5) were transfected with expression vector for constitutively active Flag-RhebQ64L (1 μg). Transfected cells were incubated under normal conditions (pH 7.4) or alkaline stress ($7.4 < pH < 8.2$) for 2 h. Western Blot analysis was performed to examine for the conversion of LC3-I into LC3-II as a measure of autophagy induction and for the phosphorylation of S6K as a measure of mTORC1 activity. B: Cells were incubated under normal conditions (pH 7.4, lane 1) or alkaline stress for 1, 2, or 4 h ($7.4 < pH < 8.7$, lanes 2–4). Cells were examined for the phosphorylation of S6K at Thr 389 by Western blot analysis. C: HeLa cells were incubated under normal conditions (pH 7.4, lane 1) or acidic stress for 1, 2, or 4 h ($7.0 < pH < 7.4$, lanes 2–4). The pHs of the media were altered to induce acidic stress by changing the ambient carbon dioxide concentration (pCO_2) from normal 5% to 20%. The pHs were measured using a calibrated pH meter. Cells were examined for the phosphorylation of S6K at Thr 389. D: HeLa cells were incubated under normal conditions (pH 7.4, lane 1) or alkaline stress ($7.4 < pH < 8.7$, lanes 2–4) for 1, 2, or 4 h. After incubation under alkaline stress for 4 h, cells were transferred to an incubator with a normal flow of 5% CO_2 and incubated for an additional 4 h (lane 5).

Inactivation of mTORC1 by alkaline stress also reduced the average size of other types of cells such as 293T and MCF7 cells (Fig. 4A). Microscopic observation indicates that the morphology of alkaline-stressed cells was basically the same as that of cells treated with rapamycin or cycloheximide. Any morphological changes to verify cell shrinkage, at least under the experimental conditions employed in our study, was not observed (Suppl Fig. 2B).

To assess whether mTORC1 inhibition by alkaline stress affects Akt pathway, phosphorylation of Akt was determined after the incubation of cells under alkaline conditions. The inactivation of mTORC1 by alkaline stress resulted in the increased Akt phosphorylations at Ser 473 and Thr 308 in HeLa cells. However, the phosphorylation of Akt was reduced to the normal level when cells were incubated in normal flow of 5% CO_2 for 4 h suggesting the reversible mTORC1 inactivation by alkaline stress (Fig. 4B).

DISCUSSION

The present study demonstrates that alkaline stress inhibits mTORC1 to induce autophagy in various types of mammalian cells. Autophagy is a multistep process including the formation of autophagic vesicles which sequesters subcellular components and maturation of autophagic vesicles. Autophagic vesicle maturation occurs through the fusion with lysosomes to form degradative autolysosomes [Eskelinen, 2005; Klionsky, 2005]. Our data in Figure 2 reveal that alkaline stress induces autophagosome maturation as well as the accumulation of autophagosomes. The carbonic acid-bicarbonate buffer was chosen for this study since it is the predominant buffer of intestinal fluids and plasma. This buffer system is unique in that the pHs can be held at desired steady state by controlling the carbon dioxide concentration (pCO_2).

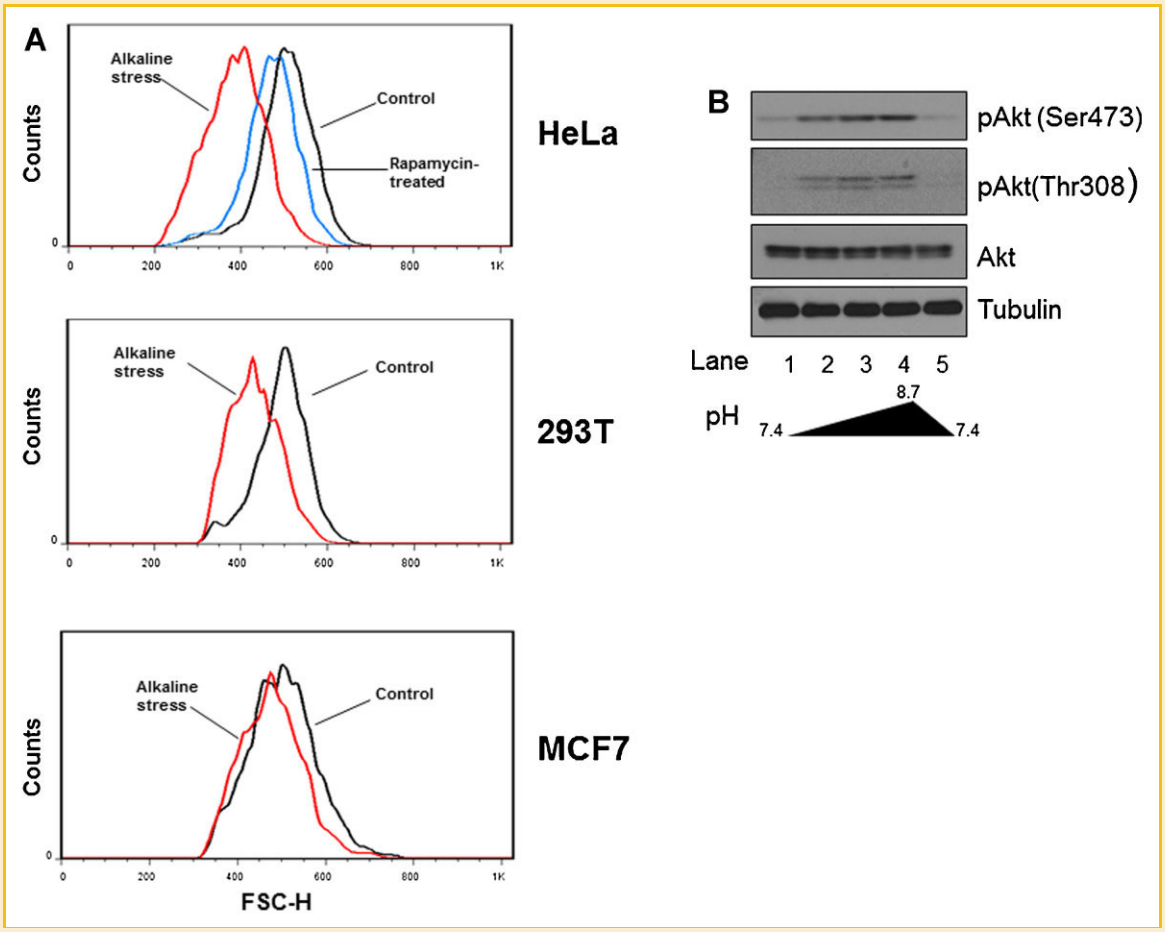

Fig. 4. Alkaline stress reduces cell size but activates Akt. A: Human cell lines including HeLa, 293T, and MCF7 were incubated under normal conditions (pH 7.4), alkaline stress ($7.4 < pH < 8.8$) for 10 h, or treated with 100 nM rapamycin for 10 h. Cells were then fixed with 70% ethanol, stained with 10 μg/ml propidium iodide (PI). Distribution of cell size was examined by flow cytometry. Representative FSC-H histograms of G1 phase cells were shown. B: HeLa cells were incubated under normal conditions (pH 7.4, lane 1) or alkaline stress ($7.4 < pH < 8.7$, lanes 2–4) for 1, 2, or 4 h. After incubation under alkaline stress for 4 h, cells were transferred to an incubator with a normal flow of 5% CO_2 and incubated for an additional 4 h (lane 5). The effect of mTORC1 inhibition by alkaline stress on Akt activation was assessed by examining the phosphorylation of Akt at Ser 473 and Thr 308.

Our data in Figure 3A showing the inhibition of alkaline stress-induced autophagy by constitutively active mutant of Rheb, RhebQ64L [Jiang and Vogt, 2008], support that the inhibition of mTORC1 activity is important for the induction of autophagy by alkaline stress. 293T cells grown under alkaline condition exhibited marked inhibition of mTORC1 as assessed by phosphorylation of S6K at Thr 389. However, autophagic pathway, which was evaluated by the conversion of LC3-II from LC3-I, was not completely inhibited by considerable activation of mTORC1 in transfected cells overexpressing RhebQ64L. One of the plausible scenarios for the incomplete inhibition of autophagy by mTORC1 activation in cells under alkaline stress is that alkaline stress-induced autophagic pathway is also activated by mTORC1-independent pathway. Autophagy has been recently described to be regulated by mTORC1-indepedent pathway in which intracytosolic levels of Ca^{2+}, phospholipase C (PLC), and cyclic AMP (cAMP) participate in the regulation of autophagy [Sarkar et al., 2009]. The partial involvement of mTORC1-independent pathway in alkaline stress-induced autophagy is also supported by the result showing higher rate of lipidated LC3-II conversion in cells treated with alkaline stress than the LC3-II conversion by the treatment of rapamycin, a known specific inhibitor of mTORC1 (Suppl Fig. 1).

How mTORC1 signaling is wired to autophagic pathway is poorly understood. However, there are some evidences implicating the essential role of mTORC1 signaling on autophagic pathway. First, autophagy can be induced by the treatment of rapamycin, a negative regulator or mTORC1 [Rubinsztein et al., 2007]. Hence, autophagic process is inhibited by replenishing nutrients mainly through the activation of mTORC1 kinase [Pattingre et al., 2008]. Second, mTORC1 downstream target genes that are essential for autophagic vesicle formation have been identified. The mTORC1 directly interacts with ULK1–mATG13-FIP200 complex, a lipid kinase signal complex that mediates autophagic vesicle nucleation. Then mTORC1 phosphorylates ULK1 and mATG13 at inhibition phosphorylation sites to inhibit ULK1 kinase activity leading to the initiation of autophagic vesicle formation [Chan, 2009; Hosokawa et al., 2009; Jung et al., 2009]. We confirmed the inhibition of mTORC1 activity

by measuring the phosphorylation of S6K in different types of human cell lines treated with alkaline stress (Fig. 3B). We found that alkaline stress triggers autophagy via complete mTORC1 inhibition. Combined treatment of cells with alkaline stress and rapamycin did not show any further induction of autophagy than the treatment of alkaline stress alone, suggesting that mTORC1 activity was completely suppressed by alkaline stress to induce autophagy (Suppl Fig. 1).

mTOR–rictor complex (mTORC2) is known to directly phosphorylate Akt at Ser 473. Thus, Akt phosphorylation status at Ser 473 has been often used to assess the activation of mTORC2. Decrease in Akt phosphorylation at Ser 473 would indicate mTORC2 inhibition. However, it is unlikely that alkaline stress inhibits mTORC2 since the phosphorylations of Akt at two phosphorylation sites were increased after alkaline stress (Fig. 4B). We could also rule out the possibility that the impairment of general regulation of acid–ase balance in cellular microenvironment inhibits mTORC1 function since acidic stress affects neither mTORC1 function nor autophagy induction in cells grown in the media adjusted to acidic pHs (Fig. 3C). The results in Figure 3D showing the restoration of mTORC1 activity imply that the inhibition of mTORC1 by alkaline stress is a reversible process unlike mTORC1 inhibition by rapamycin which has been known to irreversibly inhibit mTORC1 signaling [Balgi et al., 2009].

Data in Figure 4 showing the significant reduction in cell size and the activation of Akt further support our notion that mTORC1 activity is interfered by alkaline stress. Since mTORC1 signaling plays a crucial role on protein synthesis, mTORC1 activity is anticipated to be required for cellular growth to appropriate cell size. For example, rapamycin has been known to function in the translation control to regulate sizes of cells and organs in experimental mice [Fingar et al., 2002; Shioi et al., 2002]. Considerable reduction in cell size by alkaline stress provides strong support for mTORC1 inactivation in cells under alkaline stress (Fig. 4A). However, alkaline stress induced greater reduction of cell size in HeLa cells than rapamycin or cycloheximide does (Suppl Fig. 2A). In addition to mTORC1 signaling pathway, alkaline stress seems to affect many other metabolic pathways that regulate cell volume, cell growth, and cell proliferation.

Recent studies have revealed that mTORC1 inhibition can increase Akt phosphorylation at Thr 308 and Ser 473 due to the negative feedback loops mediated by the upregulation of IRS1 (insulin receptor substrate 1) [Harrington et al., 2005; O'Reilly et al., 2006] and the downregulation of S6K [Manning, 2004; Han et al., 2007]. The increase of Akt phosphorylation at Thr 308 and Ser 473 in cells grown under alkaline conditions advocates that the mTORC1 activity was inhibited by alkaline stress (Fig. 4B). Thus, our data showing alkaline stress increases Akt phosphorylation support that alkaline stress specifically inhibits mTORC1. Activation phosphorylation of Akt in cells undergoing alkaline stress-induced autophagy is particularly intriguing when considering that Akt is an upstream signaling that activates mTORC1. Alkaline stress inhibits mTORC1 but activates Akt by relieving the negative feedback inhibition of Akt. The underlying mechanism by which mTORC1 could remain inhibited under alkaline conditions when Akt phosphorylation is activated is unknown at present. One of the plausible scenarios for this controversial issue is that mTOR changes its state to a form that could not be activated by upstream activators under alkaline conditions. Conformation changes in the mTOR kinase domain have been known to be important for its activity [Gulati et al., 2008; Wang et al., 2009]. In fact, mTOR–raptor interaction changes the conformation of mTOR kinase domain [Kim et al., 2002]. Alkaline pH may change the conformation of mTOR or inhibit its binding with mTOR partners. These changes might cause mTORC1 to become insensitive to its upstream regulators. This notion was supported by the result showing phosphorylation activation of Akt in 4 h after alkaline stress (Fig. 4B) when mTORC1 remains inhibited as to stimulate autophagy induction (Fig. 3B).

Alkalosis, a process which raises the cellular pH, is believed to be related with various pathological and physiological complications such as impaired cerebral functions [Rose, 1994]. Although it is obvious that alkaline condition is cytotoxic and lethal, the mechanism leading to alkaline-induced cytotoxicity and cell death is unknown. This study suggests that mTORC1 inhibition and subsequent induction of autophagy by alkaline stress should be considered as the underlying mechanism of various clinical conditions associated with alkalosis.

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