Autophagic Clearance of Mitochondria in the Kidney Copes with Metabolic Acidosis

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ABSTRACT
Metabolic acidosis, a common complication of CKD, causes mitochondrial stress by undefined mechanisms. Selective autophagy of impaired mitochondria, called mitophagy, contributes toward maintaining cellular homeostasis in various settings. We hypothesized that mitophagy is involved in proximal tubular cell adaptations to chronic metabolic acidosis. In transgenic mice expressing green fluorescent protein–tagged microtubule-associated protein 1 light chain 3 (GFP-LC3), NH4Cl loading increased the number of GFP puncta exclusively in the proximal tubule. In vitro, culture in acidic medium produced similar results in proximal tubular cell lines stably expressing GFP-LC3 and facilitated the degradation of SQSTM1/p62 in wild-type cells, indicating enhanced autophagic flux. Upon acid loading, proximal tubule–specific autophagy-deficient (Atg5-deficient) mice displayed significantly reduced ammonium production and severe metabolic acidosis compared with wild-type mice. In vitro and in vivo, acid loading caused Atg5-deficient proximal tubular cells to exhibit reduced mitochondrial respiratory chain activity, reduced mitochondrial membrane potential, and fragmented morphology with marked swelling in mitochondria. GFP-LC3–tagged autophagosomes colocalized with ubiquitinated mitochondria in proximal tubular cells cultured in acidic medium, suggesting that metabolic acidosis induces mitophagy. Furthermore, restoration of Atg5-intact nuclei in Atg5-deficient proximal tubular cells increased mitochondrial membrane potential and ammoniagenesis. In conclusion, metabolic acidosis induces autophagy in proximal tubular cells which is indispensable for maintaining proper mitochondrial functions including ammoniagenesis, and thus for adapted urinary acid excretion. Our results provide a rationale for the beneficial effect of alkali supplementation in CKD, a condition in which autophagy may be reduced, and suggest a new therapeutic option for acidosis by modulating autophagy.


Metabolic acidosis (MA) is one of the clinically common complications in patients with CKD. Gradual deterioration of kidney function causes mild retention of acids, leading to substantial adverse effects including the development of mineral and bone disorders, increased muscle protein degradation, impairment of glucose tolerance, progression of kidney diseases, and, most importantly, increased mortality. In healthy individuals, the kidney (as well as lung) has an important role in the...
maintenance of systemic acid base balance by excreting acid in amounts that are equal to the extrarenal acid production. The kidney has the ability to filter and reabsorb bicarbonate, synthesize ammonium, and excrete hydrogen ions. Ammoniagenesis in the mitochondria of the proximal tubular cells (PTCs) is the catabolic process in which glutaminase catalyzes glutamine into α-keto glutarate and NH₄⁺, and plays a central role among adaptive responses to MA.

Several recent clinical trials have demonstrated that alkali supplementation can retard the progression of CKD. This finding shows that MA in itself can cause cellular dysfunction; however, there is a surprising lack of experimental knowledge on the precise mechanism of cellular dysfunction associated with chronic MA. A few prior studies have reported the changes in kidney gene or protein expression in the PTCs of chronically acid-loaded animals. These studies have provided insight into the alterations of mitochondrial metabolism that either accompany or support the increased catabolism of glutamine, suggesting that MA imposes substantial stress on mitochondria. Supporting evidence has come from the observation that mitochondria isolated from the kidneys of acid-loaded rats showed slightly lower membrane potential and efficiency in oxidative phosphorylation with mild uncoupling compared with control mitochondria, whereas no significant differences were detected in liver mitochondria. Moreover, in response to acid load, mitochondria accumulates Ca²⁺ to cope with high cytosolic Ca²⁺ influx, which in turn promotes stimulation of reactive oxygen species (ROS) generation at the level of the respiratory chain. These data lead us to speculate that constitutive renovation of mitochondria is a prerequisite for kidney tubules to cope with MA.

Macrouatophagy, hereafter referred to simply as autophagy, is a highly evolutionally conserved degradation process by which cytosolic materials (including macromolecules such as proteins and lipids) and damaged organelles are nonselectively broken down to their basic components. The association of autophagy with the pathogenesis of various disorders, such as neurodegenerative diseases, cancer, and infection, has been revealed in recent years. Kidney disease is not an exception. Because the proximal tubules are potentially energy wasting and work to degrade proteins, autophagy has been supposed to play an important role in the physiology of proximal tubules. Indeed, by administering autophagy inhibitors or by using kidney-specific autophagy-deficient mice, many studies have revealed that autophagy has a protective role against PTC damage by ischemia-reperfusion and nephrotoxic reagents, including cisplatin and cyclosporine. Recent studies have indicated that autophagosomes can degrade the mitochondria in a selective manner (mitophagy), although autophagy was long assumed to be defined as the nonselective bulk degradation of cytoplasmic long-lived proteins and organelles. When the autophagy system senses signals from damaged mitochondria, an early autophagic membrane is recruited or de novo synthesized to form a double membrane along the engulfed mitochondria, and is then degraded in lysosomes. Because mitochondria are susceptible to damage mediated by ROS, maintaining an intact population of mitochondria through quality control mechanisms appears to be essential for cell survival under conditions of pathologic stress.

On the basis of this background information, we hypothesized that autophagy in the kidney proximal tubules counteracts MA by alleviating mitochondrial stress and thereby maintaining mitochondrial homeostasis. In this study, we first examined whether MA could induce autophagy in proximal tubules in vivo and in vitro, respectively. We then analyzed mitochondrial function and morphology by using acid-loaded autophagy-deficient mice and autophagy-deficient PTC lines cultured in acidic medium, especially focusing on ammonium production, respiratory chain function, mitochondrial morphology, and mitochondrial membrane potential. In addition, we investigated whether MA could induce mitophagy.

RESULTS

MA Induces Autophagy Exclusively in PTCs In Vivo and In Vitro

To investigate the induction of autophagy in kidney proximal tubules in an acidic environment, we induced MA by administering ammonium chloride (NH₄Cl) in drinking water for 30 days in green fluorescent protein–tagged microtubule-associated protein 1-light chain 3 (GFP-LC3) transgenic mice, a mouse model in which autophagosomes are labeled with GFP-positive puncta in almost all tissues. The number of GFP-LC3 puncta increased significantly in the kidney tubular cells of the acid-loaded mice compared with those of the control mice (Figure 1A, left). Coimmunostaining with megalin, a marker of PTCs, indicated that GFP-LC3 dots localized only in the proximal tubules, but not in other kidney segments or other organs (data not shown). We examined whether an acidic condition could induce autophagy in cultured PTC lines stably expressing GFP-LC3. When cultured under an acidic condition (pH 6.5), a marked increase in GFP-positive puncta was observed (Figure 1A, right). Next we performed the SQSTM1/p62 turnover assay, in which degradation of the SQSTM1/p62 protein serves as a tracer of autophagic activation. It was demonstrated that the SQSTM1/p62 protein was degraded in a time-dependent manner when cells were cultured in an acidic medium (pH 6.5), whereas the protein level remained unchanged when cultured in a normal medium (pH 7.4) (Figure 1B). The expression of SQSTM1/p62 mRNA was unchanged when cells were cultured in acidic or normal medium (data not shown).

Furthermore, we analyzed the histologic changes in the kidneys of proximal tubule–specific autophagy-deficient mice (Atg⁵¹⁹⁻:KAP mice) after administering NH₄Cl in their drinking water for 30 days. Although periodic acid–Schiff staining revealed no apparent morphologic change between acid-loaded and vehicle-treated Atg⁵¹⁹⁻:KAP mice (Supplemental
Acid loading significantly increased urinary excretion of ammonium in control and Atg5F/F:KAP mice, whereas it was rarely observed in acid-loaded or vehicle-treated control mice (Figure 2A). We next examined the effect of autophagy on ammoniagenesis in three different kidney sections (Figure 2B). Acid loading significantly induced ammoniagenesis both in the cortex and medulla of control mice, but not in the cortex and medulla of Atg5F/F:KAP mice (Figure 2C). The difference was prominent in the outer medulla, where kidney androgen-regulated protein is predominantly expressed (Kimura et al., 15; Supplemental Figure 3). Moreover, to confirm the compensatory mechanism of autophagy for acid load in vitro, we analyzed ammoniagenesis using immortalized Atg5-deficient (Atg5-negative) kidney PTCs, and autophagy-competent (Atg5-positive) kidney PTCs as a control. Similar to in vivo analysis, acid-loaded Atg5-negative PTCs produced a lower amount of ammonia than Atg5-positive PTCs (Figure 2D). These results suggest that autophagy in the PTCs contributes to compensation for MA through assisting ammoniagenesis.

**Autophagy Protects Mitochondrial Function and Morphology from Acid Loading**

Recent cumulative evidence suggests that autophagy controls the quality of mitochondria by selective elimination of impaired mitochondria, which is called mitophagy. Given that ammonia production, which is mainly managed in...
mitochondria, is reduced in autophagy-deficient PTCs under acidic conditions, we hypothesized that mitochondrial functions might be impaired in acid-loaded autophagy-deficient mice. To examine the mitochondrial functions in vivo, we measured the enzymatic activities of cytochrome oxidase (COX) and succinate dehydrogenase (SDH) on kidney sections in situ, both of which reflect mitochondrial respiratory function (Figure 3, A and B). 27 Atg5F/F:KAP mice showed less COX and SDH staining (which was prominent under MA) compared with control mice.

We next measured the mitochondrial oxygen consumption rate (OCR) of kidney PTCs in vitro to quantitatively demonstrate the mitochondrial dysfunction under acidic conditions (Figure 3, C and D). The effect of acid loading on the OCR was assessed by comparing the response of PTCs to mitochondrial inhibitors, including oligomycin (an ATP synthase inhibitor), protonophore carbonyl cyanide 4-(trifluoromethoxy) phenyldiazzone (FCCP, a mitochondrial uncoupler), rotenone, and antimycin (inhibitors of the mitochondrial respiratory chain) (Supplemental Figure 4). The basal OCR, the ATP-linked OCR, and maximum respiratory function were significantly reduced by acid loading (Figure 3C). The reduction in the ATP-linked OCR by acid loading became even more pronounced in Atg5-negative PTCs than in Atg5-positive PTCs (Figure 3D). Together, these results indicate the reduced activity of mitochondrial respiratory activity in Atg5F/F:KAP mice under MA.

We next assessed mitochondrial morphology using electron microscopy. Acid loading resulted in severe mitochondrial fragmentation and swelling with derangement of the cristae of the Atg5F/F:KAP kidney compared with vehicle treatment, whereas there was no remarkable morphologic change in the mitochondria of either the vehicle-treated or the acid-loaded wild-type mice (Figure 4).

We further examined the mitochondrial membrane potential (Δψm) of Atg5-positive and Atg5-negative PTCs with or without acid loading using tetramethylrhodamine ethyl ester (TMRE) (Figure 5). Δψm was significantly reduced both in Atg5-positive and Atg5-negative PTCs by acid loading but the reduction was more prominent in Atg5-negative PTCs. The uptake of TMRE into mitochondria can be dependent on the intracellular concentration, which in turn is determined by the cell membrane potential. Therefore, we assessed the plasma membrane potential (Δψp) by using DiBAC4(3), a bis-oxonol-type, membrane potential–sensitive dye. It was demonstrated that there was no difference in the Δψp between Atg5-positive and Atg5-negative PTCs with or without acid loading (Supplemental Figure 5).

Collectively, PTCs in acid-loaded Atg5-deficient mice and/or Atg5-deficient cultured cells exhibited reduced mitochondrial function and distorted morphology under acidic condition.

**Acid Loading Induces Mitophagy in PTCs**

Because autophagy has been known to selectively degrade mitochondria (in the process called mitophagy), we examined
whether acid loading induces autophagy to eliminate impaired mitochondria. We estimated the extent of mitophagy by staining mitochondria with MitoTracker Red FM in GFP-LC3–transfected PTCs after treating with baflomycin A1, a specific inhibitor of the vacuolar type H^+-ATPase (Figure 6A). Representative images demonstrated that more autophagosomes were colocalized with mitochondria in cells under the acidic condition than under the normal condition (indicated by yellow dots in pseudocolor images in Figure 6A). To assess the colocalization objectively, we used the Pearson correlation coefficient as the quantification method.28 This also showed the significant difference between acidic and normal conditions (Figure 6B). Furthermore, to investigate whether ubiquitinated mitochondria were recognized as a target of autophagic degradation, we constructed GFP-LC3 and mStrawberry-ubiquitin double-transfected PTCs, and then stained mitochondria with MitoTracker Deep Red FM after arresting autophagosome degradation with baflomycin A1.
Representative images showed that more autophagosomes were localized with mitochondria and ubiquitin under the acidic condition than under the normal condition (Figure 6C). Quantification using the Pearson correlation coefficient presented the significant difference between acidic and normal conditions (Figure 6D). These results show that autophagy actively eliminates acid load–induced impaired mitochondria.

**DISCUSSION**

In this study, we provide several lines of evidence that autophagic renovation of mitochondria in PTCs copes with MA–caused cellular stresses. MA was seen to induce autophagy exclusively in the PTCs. Otherwise, mitochondrial impairment became apparent and was observed as follows: (1) ammoniagenesis in the mitochondria was blunted, (2) mitochondrial respiratory chain function was impaired, and (3) mitochondrial morphology was biased toward fission status with marked swelling. Autophagy deficiency triggers a vicious cycle in which disturbed ammoniagenesis fails to compensate for MA, which further deteriorates mitochondrial dysfunction. Notably, these findings could be unique to PTCs, because an increase of ammoniagenesis in response to acid loading was defective in other kidney cells, including M-1 collecting duct cells and mouse primary mesangial cells, and knockdown of Atg5 did not affect the level of ammonia production (Supplemental Figure 6 and data not shown). We further demonstrated that mitochondria are the major target of autophagy (Figure 8). In previous studies, MA was found to activate a series of regulatory mechanisms to compensate for disordered acid-base balance, including complement activation,30 endothelin production, and activation of the renin-angiotensin system.31–33 This subsequently induced additional kidney damage. However, in contrast with clinical consequences such as the development of a catabolic state,34 development or exacerbation of bone disease,35 and deterioration of kidney function,36 the decisive mechanism by which MA induces cellular dysfunction was largely unknown. This study unraveled the molecular

**Figure 4.** Autophagy deficiency distorts the mitochondrial morphology of the PTCs under MA. Electron microscopic images of kidney sections of control and Atg5F/F:KAP mice with or without MA are presented. F/F, Atg5F/F mice; F/F:KAP, Atg5F/F:KAP mice. Bar, 5 µm in top row; 500 nm in bottom row.

**Figure 5.** Autophagy deficiency reduces the mitochondrial membrane potential in the PTCs under MA. The mitochondrial membrane potential of Atg5-positive and Atg5-negative PTCs cultured under normal and acidic media is assessed by TRME (n=5, respectively). All values are given as the mean±SEM. *P<0.05. Atg5(+), Atg5-positive PTC; Atg5(−), Atg5-negative PTC. Bar, 50 µm.

mitochondrial donor cells originating from Atg5-negative PTCs and the nuclear donor cells originating from Atg5-positive PTCs, with a “control cybrid” as a control, by fusing the mitochondrial donor cells and the nuclear donor cells, both of which originate from Atg5-negative PTCs (Figure 7A). Although the mitochondrial membrane potential was reduced and ammoniagenesis was blunted in the control cybrid under the acidic condition, both were significantly recovered in the rescue cybrid (Figure 7, B and C). These results indicate that the rescue cybrid could eliminate the damaged mitochondria from Atg5-negative PTCs by restoring the autophagic degradation process.

**Restoring Autophagic Activity Regains Ammoniagenesis via Quality Control of Mitochondria in the Acidic Condition In Vitro**

To verify that autophagy contributes to mitochondrial homeostasis under MA, we generated a transmitochondrial cybrid, which is a useful method for studying the functional effects of mitochondria in a defined nuclear background.29 At first, we generated a “rescue cybrid” by fusing the mitochondrial donor cells originating from Atg5-negative PTCs and the nuclear donor cells originating from Atg5-positive PTCs, with a “control cybrid” as a control, by fusing the mitochondrial donor cells and the nuclear donor cells, both of which originate from Atg5-negative PTCs (Figure 7A). Although the mitochondrial membrane potential was reduced and ammoniagenesis was blunted in the control cybrid under the acidic condition, both were significantly recovered in the rescue cybrid (Figure 7, B and C). These results indicate that the rescue cybrid could eliminate the damaged mitochondria from Atg5-negative PTCs by restoring the autophagic degradation process.

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mechanism of MA-associated kidney dysfunction, thereby providing the rationale for the beneficial effect of alkali supplementation for patients with CKD.

Several prior studies have reported that, in tumor cells, extracellular acidosis resulted from hypoxic condition due to chaotic vasculature, and glycolytic phenotype induces autophagy, which serves as an adaptation mechanism for cell survival by fueling energy. In particular, elevated autophagy maintained chronically in low pH-conditioned cells allowed the cells to restore their proliferative capacity for 3 months. The contribution of activated autophagy is quite different between PTCs and tumor cells because tumor cells often downregulate mitochondrial respiration in preference to aerobic glycolysis.

Mitophagy is a selective type of autophagy in which specifically targeted mitochondria are degraded by the autophagic process, and it plays an important role in the quality control of mitochondria. Recent studies have shown that canonical (nonselective) autophagy and mitophagy share similar core autophagosome/mitophagosome-formation machinery, but that distinct steps and molecules are involved in each autophagic process. In mitophagy, defective mitochondria are recognized via specific receptors and cargos, and are targeted to autophagosomes depending on organs, cells, and developmental stages. For example, mitophagy in yeast is mediated by the mitochondrial proteins Atg32 and Uth1, which serve as the mitochondrial receptors for vacuole targeting. Mitophagy also plays an essential role in erythrocyte maturation via the outer mitochondrial membrane receptor Nix (or Bnip3l) and the autophagosome-associated protein LC3. The elimination of damaged mitochondria in mammals is mediated by a pathway composed of phosphatase and tensin homolog-induced putative protein kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin. PINK1 and Parkin accumulate on damaged mitochondria and target these organelles for autophagic degradation in a process that requires Parkin-dependent ubiquitination of mitochondrial proteins. Autophagic degradation of mitochondria has been observed in the kidney, particularly in the proximal

Figure 6. Acid loading induces mitophagy in the PTCs. (A) PTCs isolated from wild-type mice stably expressing GFP-LC3 are stained with MitoTracker Red FM after treatment with 200 nM of bafilomycin A1 2 hours before harvesting (n=5). Yellow dots in merged images represent colocalization of mitochondria and autophagosomes. (B) Colocalization is assessed by Pearson’s correlation. (C) PTCs isolated from wild-type mice stably expressing GFP-LC3 and mStrawberry-ubiquitin are stained with MitoTracker Deep Red FM after treatment with 200 nM of bafilomycin A1 2 hours before harvesting (n=5). White dots in merged images represent colocalization of ubiquinated mitochondria and autophagosomes. All values are given as the mean±SEM. *P<0.05. MTR Red FM, MitoTracker Red FM; MTR Deep Red FM, MitoTracker Deep Red FM. Bar, 5 μm.
tubules, in both physiologic\textsuperscript{15} and pathologic settings, including MA (in this study), ischemia-reperfusion injury,\textsuperscript{15} and nephropathic cystinosis.\textsuperscript{45} In a strict sense, we cannot exclude the possibility that mitochondria are “predominantly” degraded by canonical autophagy. However, the observation that ubiquitinated mitochondria colocalize with the autophagosomal protein LC3 (Figure 6), which appears to be important in the mitophagic process, suggests that mitophagy, rather than canonical autophagy, is induced by MA in the proximal tubules. Although we did not examine the expression or localization of receptor/cargo molecules such as Nix, Parkin, and PINK, the observation that GFP-positive puncta in acid-loaded and ROS production, also interdependently contribute to triggering the mitophagic machinery.\textsuperscript{54,55} Observations in the present study and from other investigative groups that MA induces mitochondrial fragmentation and swelling (Figure 4), the reduction in $\Delta \psi_m$ (Figure 5), and mitochondrial accumulation of Ca$^{2+}$\textsuperscript{7} some of which become apparent only when autophagy is inhibited, support the speculation that signaling from impaired mitochondria via MA induces mitophagy.

Although autophagy was inhibited by genetic manipulation in this study, autophagic activity could actually decline in clinical practice. One prominent example is aging. Age-associated reduction in autophagic activity has been considered...
Figure 8. The proposed mechanism of autophagic renovation of mitochondria in PTCs under acidic conditions. MA-induced damaged mitochondria are eliminated adequately in the autophagy-competent condition (left), whereas damaged mitochondria accumulates in cytoplasm in the autophagy-deficient condition (right), which leads to decompensation for MA and triggers a vicious cycle.

generated by crossing KAP-Cre transgenic mice, in which Cre recombinase is expressed under control of the KAP gene promoter, and mice bearing an Atg5 flox allele (Atg5F/F mice). KAP-Cre/CAG-CAT-Z male mice, in which β-galactosidase expression reflects Cre activity, exhibited LacZ-positive tubules in the cortex and the outer medulla, whereas LacZ-positive tubules were absent in female mice (Kimura et al.15; Supplemental Figure 3). Therefore, only male mice were used in this study. All experiments were performed using 8-week-old male mice. All animal experiments were approved by the institutional committee of the Animal Research Committee of Osaka University and were in accordance with the Japanese Animal Protection and Management Law (no. 25).

### Induction of MA
For induction of MA, mice were given 0.28 M NH4Cl+0.5% sucrose in drinking water (tap water) for 30 days. Control animals received tap water with 0.5% sucrose ad libitum. All animals had free access to standard rodent chow. To confirm induction of acidosis, the blood gas data (blood pH and bicarbonate concentration) were measured by using the RAPID Lab 348 blood gas system (Siemens, Munich, Germany).

### Histologic Analyses
Mice were transcardially perfused with 4% paraformaldehyde in PBS (pH 7.4). The kidneys were removed, sectioned and postfixed in paraformaldehyde, and then embedded in paraffin or frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA). Periodic acid–Schiff staining was performed on paraffin sections. Immunohistochemical staining for SQSTM1/p62 and ubiquitin was performed on the paraffin-embedded sections after antigen retrieval via autoclaving in 0.01 mM citrate buffer (pH 6.0) for 10 minutes at 120°C and blocking with 1% BSA (Sigma-Aldrich, St. Louis, MO) in PBS for 60 minutes. Sections were incubated with primary antibodies at 4°C overnight, and were then visualized with a horseradish peroxidase–diaminobenzidine compound (Nichirei, Tokyo, Japan). To assess GFP-positive dots, post fixation tissue was sectioned and immunostained with megalin (a PTC marker) antibody. Immunofluorescence images were collected using an Olympus FV1000-D confocal laser scanning microscope (Olympus, Tokyo, Japan). Five images were collected for each sample and then LC3 dots were counted and expressed as the number per cell. For electron microscopy, the kidneys were fixed with 2% glutaraldehyde and then LC3 dots were counted and expressed as the number per cell. For electron microscopy, the kidneys were fixed with 2% glutaraldehyde and then LC3 dots were counted and expressed as the number per cell.

### Antibodies
Antibodies used included the following: antibodies for SQSTM1/p62 (Progen, Heidelberg, Germany), ubiquitin (Cell Signaling Technology, Danvers, MA), megalin (gift from Toshimi Michigami, β-actin (Sigma-Aldrich), biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), and peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark).

### Measurement of Ammonium Concentration
The kidneys were frozen in liquid nitrogen immediately after harvesting. The frozen kidney was sectioned perpendicular to its
long axis at 5- to 10-μm thickness and divided into three adjacent sections, as shown in Figure 2B. Each sample was steeped with the reagent containing sodium phosphotungstic acid to remove extra protein. The ammonium concentration was measured with the Wako NH₃ test (Wako Pure Chemicals, Osaka, Japan). After the total ammonium concentration was determined, the samples were homogenized and the pellet was resuspended in 1 N sodium hydroxide, shaken overnight, and analyzed for total protein concentration by the Pierce protein assay reagent (Thermo Fisher Scientific, Rockford, IL) as a standard. Ammonium contents in the original tissue slices were determined as ammonium concentration per milligram of protein. Ammoniagenesis in the PTCs was assessed by the ammonium concentration per milligram of protein.

**Measurement of Mitochondrial Membrane Potential**

Cells cultured in 35-mm glass-bottom dishes were subjected to the confocal analysis. The mitochondrial membrane potential was determined by staining with 50 nM of TMRE (T669; Invitrogen, Carlsbad, CA) for 30 minutes at 37°C. Then, the fluorescent images were collected using the Olympus FV1000-D (Olympus). The intensity of TMRE was measured using ImageJ (available at http://rsbweb.nih.gov/ij/index.html; National Institutes of Health, Bethesda, MD) within five sections for a given cell and corrected fluorescence-positive areas in each section.

**Assessment of Mitophagy**

To prove mitophagy, PTCs stably expressing GFP-LC3 were treated with 200 nM of bafilomycin A1 (Wako Pure Chemicals) for 2 hours at 37°C before staining with 25 nM of MitoTracker Red FM (M22425; Invitrogen) for 15 minutes at 37°C. Colocalization of autophagosomes and mitochondria was calculated using ImageJ and represented as Pearson's correlation between fluorescence of GFP and MitoTracker Red FM. We also constructed GFP-LC3 and mStrawberry-ubiquitin double-transfected PTCs to assess the association between ubiquitin and mitophagy. GFP-LC3 and mStrawberry-ubiquitin double-transfected PTCs were treated with 200 nM of bafilomycin A1 for 2 hours at 37°C before being stained with 25 nM of MitoTracker Deep Red FM (M22426; Invitrogen) for 15 minutes at 37°C. Colocalization of autophagosomes, ubiquitin, and mitochondria was calculated using ImageJ and was represented as Pearson's correlation among GFP, mStrawberry, and MitoTracker Deep Red FM.

**Western Blot Analyses**

Western blot analysis was conducted as previously described. In brief, whole-cell lysates were extracted in lysis buffer (Cell Signaling Technology), and protein concentration was determined using the Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific). An equal amount of protein lysates were loaded in each lane and separated using 12% SDS PAGE. Then, the gels were transferred onto polyvinylidene difluoride membranes. The membranes were stained with 200 nM of bafilomycin A1 (Wako Pure Chemicals) for 2 hours at 37°C before staining with 25 nM of MitoTracker Red FM (M22425; Invitrogen) for 15 minutes at 37°C.
incubated with anti-LC3, SQSTM1/p62, and β-actin antibodies at 4°C overnight. After incubation with horseradish peroxidase–conjugated secondary antibodies, detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

**Generation of Transmitochondrial Cybrids**

A transmitochondrial cybrid is a hybrid cell generated by the fusion of a mutant mitochondrial DNA–depleted cell (rho0) with enucleated cytoplasm (the mitochondria donor) of interest. Cybrid cells were prepared by a slight modification of the method reported previously.29 In brief, we generated the nuclear donor cell, called the rho0 cell, by treatment with 50 ng/ml of ethidium bromide to deplete the mitochondrial DNA in DMEM containing 4.5 g/L of glucose, 5% of FCS, 1 mM of pyruvate, and 50 μg/ml of uridine for 6–8 weeks. The nuclear donor cell was also introduced to the puromycin-resistant gene for a selection marker. The mitochondria donor cell was generated by incubation in regular medium containing 0.5 μg/ml of actinomycin D (00393-41; Nacalai Tesque, Kyoto, Japan) overnight for enucleation. Then, the nuclear donor cell and the mitochondria donor cell were fused by treatment with 45% polyethylene glycol and incubated in regular medium with puromycin for elimination of un-fused cells. After 1–2 weeks of culture in the medium with puromycin, the cybrid clones appeared.

**Measurement of Plasma Membrane Potential**

The plasma membrane potential was determined by staining cells cultured in 35-mm glass-bottom dishes with 1 μM of bis-(1,3-dibutylbarbituric acid)-trimetheoxonol, sodium salt (DiBAC4(3), Dojindo D545; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 10 minutes at 37°C. The fluorescence images were collected using the Olympus FV1000-D at 37°C to avoid the change of intensity. The intensity of DiBAC4(3) was measured using ImageJ within five sections for a given cell and corrected fluorescence-positive areas in each section.

**Atg5 Knockdown Using RNA Interference**

The plasmid encoding Atg5-specific short hairpin RNA (shRNA) was purchased from Origene (TR500113; Rockville, MD). A corresponding plasmid encoding scrambled shRNA (TR30012; Origene) was used as a control. The shRNA-expressing plasmids were stably transfected into M-1 cells using Lipofectamine 2000 (11668-019; Invitrogen) according to the manufacturer’s instruction. Western blot analysis demonstrated that the transfection of the Atg5 shRNA expression vector inhibited Atg5 expression by 77% compared with the scramble shRNA expression vector (Supplemental Figure 6).

**Statistical Analyses**

All results are given as the mean±SEM. Statistical analyses were conducted using JMP software (SAS Institute, Cary, NC). The difference between two experimental values was assessed by the t test. Multiple-group comparisons were performed using ANOVA with post-testing using the Turkey–Kramer test. P<0.05 was considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**


23. Lemasters JJ: Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. rejuvenation Res 8: 3–5, 2005

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