Mitochondrial degradation by autophagy (mitophagy) in GFP-LC3 transgenic hepatocytes during nutrient deprivation

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Fasting in vivo and nutrient deprivation in vitro enhance sequestration of mitochondria and other organelles by autophagy for recycling of essential nutrients. Here our goal was to use a transgenic mouse strain expressing green fluorescent protein (GFP) fused to rat microtubule-associated protein-1 light chain 3 (LC3), a marker protein for autophagy, to characterize the dynamics of mitochondrial turnover by autophagy (mitophagy) in hepatocytes during nutrient deprivation. In complete growth medium, GFP-LC3 fluorescence was distributed diffusely in the cytosol and incorporated in mostly small (0.2–0.3 μm) patches in proximity to mitochondria, which likely represent preautophagic structures (PAS). After nutrient deprivation plus 1 μM glucagon to simulate fasting, PAS grew into green cups (phagophores) and then rings (autophagosomes) that enveloped individual mitochondria, a process that was blocked by 3-methyladenine. Autophagic sequestration of mitochondria took place in 6.5 ± 0.4 min and often occurred coordinately with mitochondrial fission. After ring formation and apparent sequestration, mitochondria depolarized in 11.8 ± 1.4 min, as indicated by loss of tetramethylrhodamine methylster fluorescence. After ring formation, LysoTracker Red uptake, a marker of acidification, occurred gradually, becoming fully evident at 9.9 ± 1.9 min of ring formation. After acidification, GFP-LC3 fluorescence dispersed. PicoGreen labeling of mitochondrial DNA (mtDNA) showed that mtDNA was also sequestered and degraded in autophagosomes. Overall, the results indicate that PAS serve as nucleation sites for mitophagy in hepatocytes during nutrient deprivation. After autophagosome formation, mitochondrial depolarization and vesicular acidification occur, and mitochondrial contents, including mtDNA, are degraded.

mitochondria; mitochondrial DNA; mitochondrial fission; mitophagosomes; mitophagy; preautophagic structures

AUTOPHAGY REFERS TO “self-eating” where cells degrade their cellular constituents to maintain cellular homeostasis as a normal response to stresses like starvation and in pathological conditions, including cancer, muscular disorders, neurodegenerative diseases, and pathogen infections (reviewed in Ref. 26). Glucagon released from the pancreas during fasting promotes hepatic autophagy, whereas insulin released after feeding suppresses autophagy (1, 50). Autophagy salvages amino acids, fatty acids, and other molecular building blocks essential for cell survival during nutrient deprivation. Autophagy also removes protein aggregates and unwanted or dysfunctional organelles, such as mitochondria. Prompt elimination of aged, damaged, and dysfunctional mitochondria may be important to protect cells against release of proapoptotic mitochondrial proteins, mitochondrial formation of toxic reactive oxygen species (ROS), and futile hydrolysis of ATP after uncoupling (24, 26, 48).

The term mitophagy has been introduced to describe autophagic degradation of mitochondria (24), and mitophagy is the predominant mechanism to remove superfluous and damaged mitochondria. In nonproliferating tissues like heart, brain, liver, and kidney, mitochondria turn over with a half-life of 10 to 25 days (25). Although some studies support the conclusion that autophagy, including mitophagy, occurs randomly (37), other evidence supports the concept that autophagy of mitochondria and other organelles occurs selectively. In yeast grown on methanol as a carbon source, peroxisomes proliferate. Changing the nutrient broth to glucose- or ethanol-containing medium results in specific degradation of peroxisomes by autophagy, a selective process termed pexophagy (17). Peroxin 14 is required for recognition of peroxisomes for such pexophagy (5). Similarly, withdrawal of treatment of hepatocytes with diethylhexylphthalate, a peroxisome proliferator, in the presence of protease inhibitors leads to accumulation of peroxisomes in autophagosomes, indicating selective pexophagy in mammalian cells (49).

During the postnatal period, glycogen is selectively sequestered into autophagosomes to enhance glycolytic substrate generation after interruption of transplacental nutrition (23). Autophagosomes formed postnatally contain large amounts of glycogen and rarely contain mitochondria or other organelles (21). After fasting or glucagon treatment, different cytoplasmic components were found at different times in autophagosomes, indicating ordered organelle degradation (22). In MCF-7 cells, mitophagy was induced after 30 h whereas ribosomal autophagy was dominant after 12 h of nutrient deprivation. In addition, in Uth1p deficient yeast, nutrient deprivation stimulates normal autophagy of various organelles but not mitochondria (19). However, a corresponding mammalian protein is yet to be identified. Additionally, mutation of Aup1, which is a mitochondrial phosphatase homologue localized to mitochondrial intermembrane space, decreases mitophagy in yeast (40). Parkin, BNIP3, and Atg32 are also implicated in targeting mitochondria for autophagy (15, 28, 29, 41, 51). Other work indicates that the mitochondrial permeability transition is involved in targeting mitochondria for autophagy (8, 33, 34). Taken together, these studies indicate that autophagy selectively targets different organelles in response to various stimuli and stresses.
During autophagy, phagophores (also called isolation membranes) of unknown origin form that sequester and enclose components of the cytoplasm to yield double-membrane vesicles called autophagosomes (36). Subsequently, autophagosomes fuse with late endosomes and/or lysosomes to mature into autolysosomes where degradation by lysosomal hydrolases occurs. The process of autophagy requires several evolutionarily conserved Atg (autophagy-related) proteins (47). Microtubule-associated protein 1 light chain 3 (LC3) is a mammalian homologue of yeast Atg8. Atg4B proteolytically cleaves the COOH terminus of proLC3 (LC3) to form LC3 I. Atg7 and Atg3 act to conjugate LC3 I with phosphatidylethanolamine (PE) to form LC3 II, which is recruited to forming autophagosomes (14). LC3 II remains on autophagosomes until after autophagosomal fusion with lysosomes occurs. Subsequently, LC3 II entrapped inside autophagosomes becomes degraded, whereas surface LC3 II is released and presumably recycled.

Although mammalian mitochondria are constantly renewed in nondividing cells, such as the hepatocytes of normal liver, the kinetics of mitophagy are not well characterized. Here, using hepatocytes isolated from green fluorescent protein (GFP)-LC3 transgenic mice, we show that preautophagosomal structures (PAS) serve as nucleation sites in mitophagy. Individual PAS grow to envelop and sequester whole or a part of polarized mitochondria into autophagosomes. Subsequent processing leads to vesicular acidification and degradation of mitochondrial contents, including mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Materials. Collagenase A was obtained from Roche (Penzberg, Germany); 3-methyladenine (3MA), protease cocktail, and phosphatase inhibitor cocktail from Sigma Chemical (St. Louis, MO); Lyso-Tracker Red (LTR), MitoFluor Far Red (MFFR), tetramethylrhodamine methylester (TMRM), and PicoGreen from Molecular Probes (Carlsbad, CA); bicinchoninic acid (BCA) reagents from Pierce (Rockford, IL); 4%-12% Bis-Tris gels from Invitrogen (Carlsbad, CA); nitrocellulose membranes from Whatman (Dassel, Germany); rabbit anti-LC3 antibody from MBL International (Woburn, MA); goat anti-rabbit antibody from Chemicon (Billerica, MA); and chemiluminescence reagents from GE Sciences (Buckinghamshire, UK).

Hepatocyte isolation and culture. Primary hepatocytes from wild-type and GFP-LC3 transgenic C57BL/6 male mice were isolated by a two-step collagenase perfusion, as described (20). Hepatocytes were cultured overnight in 5% CO2-95% air at 37°C on Type 1 collagen-coated dishes. Hepatocytes isolated from green fluorescent protein (GFP)-LC3 transgenic C57BL/6 male mice were isolated by two-step collagenase perfusion, as described (20). Hepatocytes were cultured overnight in 5% CO2-95% air at 37°C on Type 1 collagen-coated, 35-mm glass-bottom dishes (300,000 cells per dish) in Waymouth’s MB-752/1 growth medium (WM) supplemented with 27 mM NaHCO3, 10% fetal bovine serum, 100 mM insulin, and 100 mM dexamethasone. Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill and Medical University of South Carolina.

Loading of fluorophores and induction of autophagy. GFP-LC3 hepatocytes were incubated with 500 nM TMRM, a mitochondria potential-indicating fluorophore, or 500 nM LTR, a probe of acidic organelles, for 30 min at 37°C in WM supplemented with 25 mM Na-HEPES buffer, pH 7.4, 10% fetal bovine serum, 100 mM insulin, and 100 mM dexamethasone. In some experiments, GFP-LC3 hepatocytes were colabeled with LTR and 300 nM MFFR. In other experiments, wild-type hepatocytes were colabeled with 3 μl/ml PicoGreen, a probe for labeling mtDNA (2), and 500 nM LTR. To induce autophagy by nutrient deprivation, hepatocytes were placed in Krebs-Ringer-HEPES buffer (KRH, 115 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM KH2PO4, 1.2 mM MgSO4, and 25 mM Na-HEPES buffer, pH 7.4) plus 1 μM glucagon (KRH/G). In all experiments, one-third of the initial concentration of TMRM or LTR and PicoGreen was kept in subsequent incubation media after washes to maintain equilibrium distribution of the fluorophores.

Lasering scanning confocal microscopy. Images were collected using a Zeiss LSM 510 NLO laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) with a ×63 numerical aperture 1.4 oil immersion planapochromat objective lens. Temperature of the microscope stage was maintained at 37°C using an environmental chamber. LTR and TMRM were excited at 543 nm by a helium-neon laser attenuated to <2% of full power, and emission was collected through a 560-nm long-pass filter. GFP-LC3 and PicoGreen were excited at 488 nm by an argon laser <2% of full power, and emission was collected through a 500- to 530-nm band-pass filter. MFFR was excited at 633 nm by a helium-neon laser, and emission was collected through a 650-nm long-pass filter. For time-lapse experiments, images were acquired approximately every 1 min for up to 130 min.

Quantification of autophagic structures and mitochondrial polarization. Numbers of GFP-labeled LC3 patches (PAS), cup-shaped structures (phagophores), and rings with and without red TMRM fluorescence (polarized mitophagosomes and depolarized autophagosomes, respectively) were counted in blinded fashion in confocal images of single hepatocytes from three independent experiments from three different hepatocyte isolations. Loss of TMRM typically occurred rapidly, and mitochondria were judged depolarized after >50% of fluorescence was lost. Pixel intensity for TMRM fluorescence of individual mitochondria was assessed by selected area analysis in Photoshop CS4 (Adobe Systems, San Jose, CA). Similarly, numbers of red-fluorescing, LTR-labeled structures were also counted.

Western analysis. Hepatocytes were harvested by scraping in RIPA buffer (20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 1% sodium dodecyl sulfate, and 0.1% NP-40 containing protease and phosphatase inhibitor cocktails, as recommended by the manufacturer) and sonicated on ice using an ultrasonic cell disruptor (Misonex, Farmingdale, NY). Lysates were centrifuged at 13,000 g for 10 min at 4°C. Protein concentration was measured using a BCA procedure, as recommended by the manufacturer. Cell lysates were resolved on 4 to 15% polyacrylamide gels and electrotransferred onto the nitrocellulose membranes. After blocking with 5% nonfat milk in TBST (10 mM Tris-HCl buffer, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 1 h, membranes were immunoblotted with anti-LC3 antibody diluted 1:1,000 in TBST. Primary antibody was detected with a horseradish peroxidase-conjugated anti-rabbit secondary antibody using a chemiluminescence kit according to the manufacturer’s instructions. A nonspecific band was used to confirm equal loading of protein.

Statistical analysis. Results are plotted as means ± SE. Statistical analysis was performed by Student’s t-test or ANOVA using SigmaStat 3.1 (Systat Software, Chicago, IL) with P < 0.05 as the criterion of significance.

RESULTS

Nutrient deprivation stimulates autophagosome formation in cultured GFP-LC3 hepatocytes. To evaluate the dynamics of autophagy, we examined hepatocytes isolated from GFP-LC3 transgenic mice. When GFP-LC3 hepatocytes were incubated in WM, GFP-LC3 fluorescence was distributed relatively diffusely throughout the cells with a slight concentration in the nucleus. Some GFP-LC3 fluorescence was present in small patches mostly of 0.2 to 0.3 μm in diameter (Fig. 1A, left, arrowheads) and occasional rings ~1 μm in diameter (Fig. 1A, left, double arrow). After 90 min in nutrient-free KRH plus 1 μM glucagon (KRH/G), the number of GFP-LC3 patches decreased, whereas GFP-labeled cup-shaped structures (Fig. 1,
and the intensity of LC3 II bands was very faint (Fig. 1, A). Remained unchanged after 5, 20, 50, and 90 min of incubation, by Western analysis. In WM, the intensity of LC3 I bands (18 kDa) and II (16 kDa) protein expression was then determined or KRH/G with and without 3MA for 0 to 90 min. LC3 I and LC3 II protein expression was assessed in cell extracts by Western blotting, as described in MATERIALS AND METHODS.

During autophagy, LC3 I is processed to PE-conjugated LC3 in transgenic mouse hepatocytes. A: hepatocytes from green fluorescent protein (GFP)-microtubule-associated protein-1 light chain 3 (LC3) mice were incubated either in Waymouth’s growth medium (WM) or in nutrient-free Krebs-Ringer-HEPES buffer plus glucagon (KRH/G) to stimulate nutrient deprivation. Images were collected after 90 min by laser-scanning confocal microscopy. In WM, the majority of GFP-LC3 fluorescence was diffuse in the cytosol and nucleus or incorporated into green patches [pseudophagic structures (PAS), arrowheads] and rarely in disks (autophagosomes, double arrows). In KRH/G, GFP-LC3 fluorescence appeared in numerous rings and disks (autophagosomes, double arrows) and cup-shaped structures (phagophores, arrows). Insets: magnified images of autophagic structures. B: hepatocytes from wild-type mice were incubated in WM or KRH/G for 0 to 90 min, and LC3 I and LC3 II protein expression was assessed in cell extracts by Western blotting, as described in MATERIALS AND METHODS.

These findings confirm previous studies showing induction of autophagy in this model (8, 34).

GFP-LC3-labeled phagophores sequester polarized mitochondria during nutrient deprivation. To investigate mitophagy during nutrient deprivation, hepatocytes from GFP-LC3 mice were loaded with TMRM, a red-fluorescing cationic fluorophore that accumulates electrophoretically into mitochondria and labels individual mitochondria with bright red fluorescence (Fig. 2A). Images of the hepatocytes were taken after 90 min in WM, KRH/G, or KRH/G in the presence of 3MA. In WM, a few green rings were present, but GFP-LC3 was predominately observed as small green patches or PAS (Fig. 2A, left). In KRH/G, GFP-LC3 patches decreased in number, as GFP-labeled cup-shaped structures, rings, and disks increased (Fig. 2A, middle). Frequently, TMRM-labeled polarized mitochondria occupied the interior of GFP-labeled rings, disks, and cup-shaped structures (Fig. 2A, middle), 3MA virtually completely suppressed sequestration of polarized mitochondria into GFP-LC3-labeled structures during incubation in KRH/G. In the presence of 3MA, GFP-LC3 remained as diffuse fluorescence and small patches (Fig. 2A, right).

The distribution of GFP-LC3 into various structures was quantified from images of random fields from three indepen-
dent experiments (Fig. 2B). Figure 2C illustrates the GFP-LC3-labeled autophagic structures that were scored: patches (PAS, arrowhead), cups (phagophores or isolation membranes, arrow), and rings and disks. On the basis of TMRM labeling, rings and disks were categorized as TMRM positive (polarized autophagosomes, double arrow) and TMRM negative (depolarized autophagosomes and autolysosomes, double arrowhead). After 90 min in WM, GFP-LC3 was predominantly diffuse or incorporated into PAS patches dispersed throughout the cytosol. In KRH/G, PAS patches decreased 69% compared with WM. In addition, PAS patches were frequently located near polarized mitochondria in KRH/G (Fig. 2C, arrowhead). At the same time, phagophores, polarized mitophagosomes, and depolarized autophagosomes/autolysosomes increased 1.55-, 11.1-, and 8.1-fold, respectively. Overall, ~40% of rings and disks contained TMRM-labeled mitochondria (Fig. 2B), although time-lapse imaging showed that many of the depolarized autophagosomes did initially contain polarized mitochondria (see below). 3MA virtually completely inhibited formation of phagophores, polarized mitophagosomes, and depolarized autophagosomes/autolysosomes after 90 min in KRH/G with retention of a similar number of PAS patches as in WM. Together, these data suggest that GFP-LC3 patches likely serve as nucleation sites that grow into phagophores that engulf polarized mitochondria and hence may appropriately be called PAS.

**GFP-LC3-labeled mitophagosomes mature into autolysosomes.** Newly formed autophagosomes fuse with lysosomes to mature into acidic autolysosomes. To characterize intravesicular acidification after autophagic induction by nutrient deprivation, GFP-LC3 hepatocytes were loaded with LTR, a red-fluorescing weak base that accumulates into acidic compartments. In WM, small LTR-labeled red structures of ~0.2 μm in diameter were present that presumably corresponded to primary lysosomes and late endosomes (Fig. 3A, left). These LTR-labeled vesicles did not colocalize with GFP-LC3. After 90 min incubation in KRH/G, larger LTR-labeled structures increased dramatically in number, whereas small LTR-labeled vesicles decreased (Fig. 3A, middle). Numerous LTR disks were located inside GFP-LC3 rings (Fig. 3C). Although GFP-LC3 cup-shaped structures increased after nutrient deprivation, cup-shaped structures never colocalized with LTR. After 90 min in KRH/G plus 3MA, these nutrient deprivation-induced changes were prevented, and images were virtually indistinguishable from those obtained for hepatocytes incubated in WM (Fig. 3A, right).

The distribution of LTR-labeled structures was quantified from random fields from three independent experiments. LTR-labeled acidic structures were scored as red patches (~0.2 μm, primary lysosomes/acidified late endosomes) (Fig. 3C, arrowhead), GFP-LC3 rings containing red LTR fluorescence (acidified autophagosomes) (Fig. 3C, double arrow), and solid red disks (autolysosomes) (Fig. 3C, double arrowhead). After 90 min in WM, LTR was predominantly taken up by primary lysosomal patches scattered through the cytosol (Fig. 3B). After 90 min in KRH/G, such primary lysosomes decreased 74% compared with WM. At the same time, autophagosomes and autolysosomes increased 11.2- and 9.9-fold, respectively. 3MA virtually completely prevented these changes from occurring in KRH/G. Overall, these data are consistent with the conclusion that small primary lysosomes/late endosomes participate in autolysosome formation.

**After autophagic sequestration, mitochondria depolarize and mitophagosomes acidify.** To investigate the dynamics of mitophagosome formation and processing, time-lapse images were collected of GFP-LC3 hepatocytes loaded with TMRM. Mitophagy began with growth of GFP-LC3 patches (PAS, arrowheads) into GFP-LC3 cups (Fig. 4A, arrows). Cup-shaped structures then enveloped and sequestered individual TMRM-labeled mitochondria, resulting in the formation of GFP-labeled rings. The time for transformation of PAS to fully formed phagophores averaged 3.3 ± 0.3 min (n = 28 events), whereas the time for maturation of phagophores to rings was 3.5 ± 0.4 min (n = 28 events). In favorable sections viewing phagophore formation laterally, depolarization of mitochondria (loss of TMRM fluorescence) seemed to occur coordinately with ring closure (Fig. 4A, double arrow). More often, mitophagosome formation was observed obliquely or end-on. Thus, not all rings represented closed vesicles, and TMRM fluorescence was frequently observed inside GFP-LC3 rings (Fig. 4B, double arrow). Whether such TMRM-labeled GFP-LC3 rings represented truly closed vesicles could not be determined. On average, TMRM fluorescence was lost 11.8 ± 1.4 min (n = 11 events) after apparent ring formation (Fig. 4B, double arrowheads). TMRM pixel intensity was measured from images of

![Fig. 3. Acidification of autophagosomes during nutrient deprivation plus glucagon in GFP-LC3 hepatocytes.](http://ajpcell.physiology.org/2011/10.1152/ajpcell.00781.2010)
mitochondria undergoing mitophagy in relation to TMRM intensity to mitochondria not undergoing mitophagy. Changes of TMRM intensity were not observed prior to sequestration or during the maturation of phagophores into autophagosomes (Fig. 4C). Overall, the data indicated that mitochondrial depolarization occurred at or after completion of sequestration (see also Fig. 6). Supplemental Figs. S1–S3 are movies also illustrating these phenomena. (Supplemental Material for this article is available online at the Journal website.)

In other experiments, time-lapse images of GFP-LC3 hepatocytes loaded with LTR were collected during incubation in KRH/G. Here in the absence of TMRM, PAS again grew into phagophores and then into rings with a time course similar to that observed for TMRM-labeled hepatocytes (Fig. 5A). LTR did not localize to PAS and phagophores. Rather, LTR uptake occurred only after ring formation. The time after ring formation that LTR began was difficult to assess because uptake began very gradually and imperceptibly (Fig. 5A, top right and bottom left). In the example in Fig. 5A, LTR uptake seemed to begin 6 to 8 min after GFP-LC3 ring formation. However, once clearly evident, LTR uptake then proceeded relatively rapidly, leading to maximal LTR uptake in another 2

Fig. 4. Progression of autophagic sequestration of polarized mitochondria. A and B: TMRM-loaded GFP-LC3 hepatocytes were incubated in KRH/G, and time-lapse images of TMRM-labeled mitochondria were collected every minute. Representative images are shown. In favorable views along the long axis of forming phagophores, note progression from a GFP-LC3 (green)-labeled PAS patch (arrowheads) to a cup-shaped phagophore (arrows) to a ring-shaped mitophagosome (double arrows). Loss of red TMRM fluorescence (mitochondrial depolarization) occurred at or after ring closure (double arrowheads). C: TMRM fluorescence intensity of mitochondria during the progression of mitophagy is plotted. Baseline is background-subtracted TMRM fluorescence of cellular mitochondria not in association with GFP-LC3 normalized to 100%. Other fluorescence values are for same cell mitochondria in association with PAS, phagophores, and GFP-LC3 rings before and after the beginning of mitochondrial depolarization. *P < 0.05 compared with other groups; n = 4 per group.

In some instances, GFP-LC3 developed into phagophores that did not sequester polarized mitochondria (data not shown). Such events were the minority (14.7%) with the remainder of sequestrations involving polarized mitochondria (85.3%). Consistently, the mitophagic process during nutrient deprivation began from single GFP-LC3 patches in association with target mitochondria. Moreover, as autophagy progressed, the number of GFP patches in the cytoplasm decreased (Fig. 2B). These data support the conclusion that GFP-LC3 patches are indeed PAS that act as nucleation and initiation sites for mitophagy.

Fig. 5. Acidification of autophagosomes. A: LTR-loaded GFP-LC3 hepatocytes were incubated in KRH/G, and time-lapse images were collected every minute. Double arrows indicate the formation and maturation of an autophagosome. In this example, autophagosome formation (GFP-LC3 ring) was complete at 29 min and was followed by vesicular acidification (LTR uptake) beginning between 35 and 37 min. Acidification (LTR uptake) became maximal at ~42 min. B: GFP-LC3 hepatocytes were loaded with LTR and MitoFluor Far Red (MFFR) and incubated 90 min in KRH/G. Arrows, double arrows, arrowheads, and double arrowheads indicate a polarized mitophagosome, a later depolarizing mitophagosome, an autolysosome still containing GFP-LC3 fluorescence, and a later autolysosome that has lost remnants of GFP-LC3, respectively.
to 3 min. Overall, fully evident LTR accumulation indicating acidification was observed after an average of 9.9 ± 1.9 min (n = 28) following apparent GFP-LC3 ring formation (Fig. 5A). Subsequent to strong LTR labeling of autophagosomes, GFP-LC3 fluorescence was frequently lost altogether (Fig. 5A, bottom right).

To confirm that GFP-LC3-labeled mitophagosomes matured into LTR-loaded autolysosomes, GFP-LC3 hepatocytes were coloaded with MFFR to monitor mitochondrial membrane potential and LTR to monitor acidification and then incubated in KRH/G for 90 min. Similar to our observations with TMRM-labeled mitochondria (Fig. 2A), GFP-LC3 formed phagophores that engulfed MFFR-labeled mitochondria (pseudo-colored blue) to form nonacidified polarized mitophagosomes (Fig. 5B, arrow). Other presumably more mature mitophagosomes retained less MFFR fluorescence (Fig. 5B, double arrow). Fusion of primary lysosomes then led to acidified autophagosomes still containing remnants of GFP-LC3 (Fig. 5B, arrowhead), which were then lost (Fig. 5B, double arrowhead). Unlike TMRM, some MFFR was sometimes retained in late mitophagosomes and autolysosomes, suggesting nonspecific binding of MFFR to mitochondrial components or slow redistribution of MFFR after changes of membrane potential. Together, these data indicate that mitophagosomes mature into acidified autolysosomes.

Mitochondrial fission occurs coordinately with mitophagy. Frequently, phagophores sequestered portions of individual mitochondria rather than whole mitochondria into mitophagosomes (Fig. 6). This mitochondrial fission occurred as LC3-labeled rings formed, as if phagophores were pinching off portions of mitochondria. Fission events during mitophagy occurred from both the middle (Fig. 6A) and ends of individual mitochondria (Fig. 6B). Fission during mitophagy from middles and ends occurred 9.1% and 43.1% of the time, respectively, with sequestration of whole mitochondria occurring in the remainder. The intensity of TMRM fluorescence did not appear to differ between the sequestered and nonsequestered portions of mitochondria being targeted for mitophagy (see Fig. 4C). Mitochondria depolarized only after fission and ring formation were complete. Overall, these findings indicated that mitochondrial fission occurred in close coordination with autophagosome formation. Supplemental Figs. S3–S5 are movies also illustrating mitochondrial fission occurring coordinately with mitophagy.

Mitophagy sequesters and degrades mitochondrial DNA. Mitochondria are a major source of ROS, which can lead to mutations in mtDNA. mtDNA lacks histones, and mitochondria have limited DNA repair capacity compared with the nucleus, which makes mtDNA more vulnerable to oxidative damage (45). To investigate a role of mitophagy in mtDNA degradation and turnover, hepatocytes from wild-type mice were colabeled with green-fluorescing PicoGreen and red-fluorescing TMRM or LTR. PicoGreen is a DNA-intercalating fluorophore that penetrates mitochondrial membranes and labels mtDNA. In hepatocytes incubated in WM, virtually every TMRM-labeled mitochondrion contained PicoGreen-labeled mtDNA nucleoids, which were yellow in the overlay (Fig. 7A). Virtually no PicoGreen-labeled structures were present in the cytoplasm that did not colocalize with mitochondria. Hepatocytes were also colabeled with PicoGreen and LTR. After only 1 min in KRH/G, almost no PicoGreen-labeled mtDNA nucleoids colocalized with LTR (Fig. 7B, left, arrows). Similarly, for hepatocytes in WM, very little colocalization of LTR and mtDNA was observed (data not shown). By contrast, after 120 min in KRH/G, PicoGreen-labeled structures were less numerous and now present in some LTR-labeled vesicles (Fig. 7B, arrows). Time-lapse confocal imaging showed envelopment of mtDNA nucleoids by LTR as mitophagosomes formed during incubation in KRH/G (Fig. 7C, arrows; see also Supplemental Fig. S6). Subsequently, after vesicular acidification, the intensity of PicoGreen decreased, indicating degradation of mtDNA. Overall, these results showed that individual polarized mitochondria in hepatocytes contain multiple copies of mtDNA in distinct nucleoids. During mitophagy, mitochondria and this mtDNA are sequestered into mitophagosomes, which acidify and then degrade the mtDNA. Thus, mitophagy is an important mechanism for mtDNA degradation and turnover.

DISCUSSION

Mitophagy is suggested to be a major degradation mechanism by which mitochondria turn over. In hepatocytes during nutrient deprivation, mitochondria are degraded at a rate of ~2.5% per hour and ~20% of mitochondria are degraded after 12 h (8). However, the dynamics of mitophagy of individual hepatic mitochondria have not been well studied. Here, we investigated mitophagy in GFP-LC3-expressing mouse hepa-
mitochondria that appear yellow in the overlay. Incubated in WM. Note one to several mtDNA nucleoids inside individual mitochondria decreased and virtually disappeared. Sequestered mtDNA was later incorporated into small patches of mostly 0.2 to 0.3 μm in diameter. Some concentration of GFP-LC3 was also observed in the nucleus, and occasional GFP-LC3 rings characteristic of autophagosomes were also present in complete growth medium (Fig. 1). The small patches likely represented PAS, as described in yeast. PAS contain complexes of Atg proteins forming a perivacuolar structure in yeast (16, 38, 39). PAS elongate into cup-shaped phagophores and then close off into autophagosomes.

The origin of phagophores has been controversial. From some studies, ribosome-free regions of the endoplasmic reticulum (ER) or Golgi complex are proposed as the source of these membranes (7, 12, 46). However, the lipid and protein composition of autophagosomal membranes differs from membranes of the ER and Golgi complex (16). Other studies suggest that isolation membranes originate from a novel membranous structure that is neither ER nor Golgi (10). A more recent speculation is that the membranous structure of isolation membranes is donated from the organelle to be sequestered (38). Membranous structure that is neither ER nor Golgi (10). A more recent speculation is that the membranous structure of isolation membranes is donated from the organelle to be sequestered (38).

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across membranes after changes of membrane potential. Redistribution time depends both on the probe and the vesicle surface-to-volume ratio, which is high for the cristae-containing mitochondrial inner membrane and lower for simple autophagosomes (35, 44). Overall, loss of TMRM fluorescence occurred in 11.8 ± 1.4 min after the first appearance of rings, but the time of depolarization after complete closure of the forming autophagosomes is likely less. Nonetheless, mitochondrial depolarization appeared to occur at or after the completion of autophagic sequestration. Thus, mitochondrial depolarization itself was not the signal initiating closure of phagophores and completion of autophagic sequestration. Also, we saw no evidence of partial mitochondrial depolarization prior to autophagic sequestration.

After ring formation, acidification occurred gradually at first and then more rapidly to become fully evident after 9.9 ± 1.9 min as assessed by LTR uptake in time-lapse images (Fig. 5A). In a previous study, the lifetime of LTR-labeled autolysosomes was measured to be ~9 min by time-lapse confocal imaging (34, 35). This lifetime of autolysosomes is difficult to assess precisely, since degradation of autolysosomes cannot be easily distinguished from movement of autolysosomes out of the confocal plane. However, electron microscopic morphometry also indicates a half-lifetime for autophagosomes of ~9 min in liver after autophagy is suppressed with insulin (30).

During nutrient deprivation-induced mitophagy, often only a portion of an individual mitochondrion became sequestered into an autophagosome because of concurrent and apparently well-coordinated mitochondrial fission (Fig. 6). Overall, fission occurred in about half of autophagic events with both ends and middle portions of mitochondria becoming sequestered. As judged by the intensity of TMRM fluorescence, mitochondrial membrane potential of the sequestering and nonsequestering parts was not different (Fig. 6C). These findings show that mitochondrial fission often occurs coordinately with autophagosome formation.

Cell death and signs of cellular stress were negligible after incubation up to 2 h in nutrient-free KRH (data not shown), consistent with our previous studies of hepatocytes showing virtually no nuclear labeling with propidium iodide or Trypan blue, little release of lactate dehydrogenase, no cell shrinkage and detachment, no necrotic or other blebbing, no TUNEL, and no mitochondrial depolarization (18, 31). Additionally, after nutrient deprivation in vivo (fasting) of GFP-LC3 transgenic mice, autophagy is strongly upregulated, but negligible hepatocellular death occurs (27).

In a recent report appearing after the present work was completed, mitochondrial fission and autophagy were characterized in Ins1 insulinoma cells (43). Mitochondrial fission and partial depolarization preceded mitophagy, as assessed by accumulation of MitoTracker Red-labeled mitochondria into GFP-LC3-labeled structures in the presence of protease inhibitors. Mitochondria undergoing mitophagy contained decreased optic atrophy type 1 (Opa1), a fusion-promoting protein in mitochondria. Moreover, overexpression of Opa1 and suppression of fission-1 (Fis1) or dynamin-related protein 1 (Drp1), two proteins involved in mitochondrial fission, decreased mitophagy. Thus, mitochondrial fission promoted mitophagy, whereas fusion was inhibitory. However, in contrast to the present work in hepatocytes in which mitochondrial fission, depolarization, and autophagic sequestration took place in only a few minutes, mitochondrial fission and depolarization occurred hours before autophagy in Ins1 cells. This striking difference in time course may be due to differences of conditions (nutrient deprivation vs. nutrient replete incubation), cell type (hepatocytes vs. Ins1 cells), and methodologies.

How mitochondrial fission and mitophagy are coordinated remains to be determined. One possibility is that Atg proteins become directly involved in mitochondrial fission. For example, Atg5–Atg12/Atg16 protein complexes, which function in forming sequestering membranes, may also assist in dividing mitochondria. Atg9 is suggested to supply lipids for autophagosomal membranes and may act to insert lipids into mitochondria membranes to assist in fission (32, 38). Another possibility is that LC3 and other Atg proteins collaborate with mitochondrial fusion and fission proteins, such as mitofusin 1 (Mfn1) and Drp1. In primary neurons, mitochondrial fission induced by nitric oxide was accompanied by mitophagy (4). This fission process was inhibited by Mfn1 and dominant negative Drp1 expression. Future experiments will be needed to define the molecular events involved in coordinating mitophagy with mitochondrial fission.

On average, mitochondria of hepatocytes contain four to five copies of mtDNA (6). In complete growth medium, PicoGreen labeling confirmed that each polarized mitochondrion of hepatocytes contained multiple copies of mtDNA in distinct nucleoids (Fig. 5A). After autophagic induction, PicoGreen-labeled mtDNA moved into autolysosomes as mitophagy occurred (Fig. 5, B and C). Thus, mtDNA was not excluded from mitochondria undergoing mitophagy. After maximal autolysosomal acidification as indicated by LTR uptake, PicoGreen fluorescence gradually disappeared, consistent with previous reports of mtDNA degradation by DNase II of lysosomes (9, 11). mtDNA has a 10- to 20-fold higher mutation rate than nuclear DNA due to the proximity of mtDNA to the ROS-generating respiratory chain, the limited DNA repair capacity of mitochondria, and the lack of protective histones in mtDNA (45). Mutations of mtDNA lead to mitochondrial dysfunction due to absent or abnormal synthesis of one or more of the 13 protein subunits of oxidative phosphorylation that are encoded by mtDNA. Defective mtDNA in mice leads to shortened life span and earlier onset of alopecia, osteoporosis, kyphosis, anemia, and reduced fertility, characteristic traits of aging in mice and other species, including humans (42). Our study indicates that mitophagy is a major pathway for mtDNA degradation and supports that proposal that mitophagy plays an important role in elimination of damaged and mutated mtDNA in cells (24).

In summary, nutrient deprivation accelerated mitophagy in cultured rat hepatocytes. Since mitochondria were sequestered in 85% of autophagosomes but comprise only 20% of the volume of the cytosol, nutrient deprivation-induced autophagy would appear to involve, at least in part, selective mitophagy. In this mitophagy, PAS first associated with polarized mitochondria and in a few minutes grew into cup-shaped phagophores that enveloped and sequestered target mitochondria in autophagic vesicles. Frequently, mitophagy occurred coordinately with mitochondria fission. Abrupt mitochondrial depolarization occurred at or following sequestration. Lastly, the fully formed mitophagosomes acidified and their contents, including mtDNA, degraded. These events provide a framework for analyzing mitophagy in disease.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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