Increased levels of reduced cytochrome $b$ and mitophagy components are required to trigger nonspecific autophagy following induced mitochondrial dysfunction

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Accepted 21 November 2012

Journal of Cell Science 126, 415–426
2013. Published by The Company of Biologists Ltd
doi: 10.1242/jcs.103713

Summary

Mitochondria are essential organelles producing most of the energy required for the cell. A selective autophagic process called mitophagy removes damaged mitochondria, which is critical for proper cellular homeostasis; dysfunctional mitochondria can generate excess reactive oxygen species that can further damage the organelle as well as other cellular components. Although proper cell physiology requires the maintenance of a healthy pool of mitochondria, little is known about the mechanism underlying the recognition and selection of damaged organelles. In this study, we investigated the cellular fate of mitochondria damaged by the action of respiratory inhibitors (antimycin A, myxothiazol, KCN) that act on mitochondrial respiratory complexes III and IV, but have different effects with regard to the production of reactive oxygen species and increased levels of reduced cytochromes. Antimycin A and potassium cyanide effectively induced nonspecific autophagy, but not mitophagy, in a wild-type strain of *Saccharomyces cerevisiae*; however, low or no autophagic activity was measured in strains deficient for genes that encode proteins involved in mitophagy, including *ATG32*, *ATG11* and *BCK1*. These results provide evidence for a major role of specific mitophagy factors in the control of a general autophagic cellular response induced by mitochondrial alteration. Moreover, increased levels of reduced cytochrome $b$, one of the components of the respiratory chain, could be the first signal of this induction pathway.

Key words: Antimycin A, Autophagy, Mitochondria, Mitophagy, Yeast

Introduction

Mitochondria are central organelles for the energy metabolism of eukaryotic cells and are crucial for numerous metabolic pathways, including amino acids biosynthesis, lipid catabolism, and heme and iron-sulfur cluster synthesis. They also play key roles in the regulation of programmed cell death (Neupert, 1997; Newmeyer and Ferguson-Miller, 2003; Green and Kroemer, 2004; Lill and Müllenhoff, 2005). The maintenance of functional organelles, however, is costly in terms of cellular energy, whereas dysfunctional organelles can result in significant damage to the cell. Thus, mitochondrial biogenesis and degradation must be tightly regulated to prevent the mitochondrial response to changes in energetic demand, cellular metabolism, or environmental conditions, and to allow the removal and replacement of damaged organelles (Liu and Butow, 2006; Ryan and Hoogenraad, 2007). Consequently, not only the biogenesis but also the degradation of the mitochondrial compartment are crucial processes for the proper metabolism of the eukaryotic cell.

Autophagy is a highly conserved catabolic process in eukaryotic cells, responsible for the degradation of complex structures and long-lived proteins. Autophagy is switched on in part by the inhibition of the universally conserved protein-kinase target of rapamycin (TOR) (Noda and Ohsumi, 1998). The process of autophagy plays a pivotal role in cellular physiology by recycling cytoplasmic components in response to different stresses such as nutrient or growth factor depletion, and is important for cellular remodeling, development and differentiation. The levels of autophagy must be precisely controlled to prevent cellular dysfunction because either too little or too much autophagy can be detrimental to cellular physiology. Autophagy is associated with neurodegenerative diseases, cancer, pathogen infection and myopathies (Deretic and Levine, 2009; Banerjee et al., 2010; Tolkovsky, 2010; Alirezaei et al., 2011; Menzies et al., 2011; Rosenfeldt and Ryan, 2011). Based on morphological and mechanistic characteristics, three types of autophagy are defined: chaperone-mediated autophagy, macroautophagy (the most frequent and best described form) and
microautophagy. Chaperone-mediated autophagy is quite distinct from the other types of autophagy, and requires its protein substrates to be unfolded prior to direct translocation across the lysosome limiting membrane; accordingly, this process is not involved in the elimination of organelles. Macrautophagy involves the sequestration of portions of the cytoplasm, including entire organelles, through double-membrane structures, termed autophagosomes, and their degradation in lysosomes (mammalian cells) or in vacuoles (fungi and plants). During microautophagy, cytosolic components are engulfed directly by lytic organelles through invaginations of the lysosomal or vacuolar membrane. In yeast, both macro- and microautophagy can be morphologically characterized by the presence of ‘autophagic bodies’, which are intravacuolar vesicles containing cytosolic material.

Macro- and microautophagy can be nonselective processes that sequester bulk cytoplasm, and this is the likely outcome during a starvation response. However, the selective autophagy of damaged or excess organelles, such as peroxisomes (Farre` and Subramani, 2004), endoplasmic reticulum (Bernales et al., 2007), the nucleus (Roberts et al., 2003) and mitochondria (Kiššová et al., 2004; Rodriguez-Enriquez et al., 2004), or specific cytoplasmic components such as ribosomes (Kraft et al., 2008) occurs through micro- and macroautophagy, under certain conditions. The existence of a selective process of autophagy targeting mitochondria, called mitophagy, has been postulated on the basis that cells have to remove and replace altered mitochondria to maintain a pool of optimally functional organelles (Tolkovsky et al., 2002). It has further been proposed that alterations of this ‘housekeeping’ process would be involved in cellular aging (Lemasters, 2005). Takeshige et al. report the presence of mitochondria in the vacuoles of glucose-grown cells submitted to nitrogen starvation (Takeshige et al., 1992). However, mitochondria can be randomly sequestered in autophagosomes, like any cytoplasmic component. The utilization of the non-fermentable carbon source lactate allowed us to provide the first demonstration that mitochondria could be selectively targeted by an autophagic process distinct from the general nonselective process (Kiššová et al., 2004). This mitochondria-selective process is strictly dependent on the presence of the mitochondrial protein Uth1, whereas the nonselective process related to microautophagy still occurs in Uth1-deficient strains (Kiššová et al., 2004; Kiššová et al., 2007). Although Uth1 is not needed for mitophagy under all nutrient conditions, the characterization of this protein’s role provided the first experimental evidence that the molecular mechanisms underlying selective mitophagy are, at least partially, distinct from those underlying nonselective macro or microautophagy. Furthermore, stationary-phase yeast cells undergo mitophagy in a pathway that requires the Ptc6/Aup1 phosphatase (Tal et al., 2007). More recently, two mitochondrial proteins, Atg32 and Atg33, and the adaptor protein Atg11, have been identified as necessary components for mitophagy (Okamoto et al., 2009; Kanki et al., 2009a; Kanki et al., 2009b). Altogether, these data have established the existence of a mitochondria-selective autophagic process in yeast cells.

Considering the significant role of mitochondria in many processes, ranging from energy production to cell death, the regulation of mitophagy is likely to be tightly controlled. Mitophagy occurs in response to various stimuli, both in yeast and in mammalian cells. Until now, the main role of mitophagy has been tentatively defined as a housekeeping process, which allows the degradation of altered/dysfunctional mitochondria. However, mitophagy may also have a function in adapting the number and the quality of mitochondria to new environmental conditions. In either case, it is not known how the cell selectively targets a subset of the mitochondrial pool for degradation.

Because of the central role of mitochondria in many biological processes and pathological issues, the possible role of mitophagy in the cellular response to different types of cellular alterations, has been the focus of much research. Experiments in mammalian cells show that mitochondria can be actively degraded after the interruption of apoptosis (Tolkovsky et al., 2002; Xue et al., 1999; Xue et al., 2001) or upon mitochondrial permeability transition pore opening (Rodriguez-Enriquez et al., 2004; Kim et al., 2007). Furthermore, the inactivation of mitochondrial catalase induces the autophagic elimination of altered mitochondria (Yu et al., 2006). In yeast, alterations of mitochondrial biogenesis (Priault et al., 2005) or alterations of the maintenance of ionic balance (Nowikovsky et al., 2007) trigger mitophagy and autophagy. Conversely, recent findings have suggested that autophagy-deficient mutants accumulate altered mitochondria (Zhang et al., 2007; Suzuki et al., 2011).

It is well established that mitochondrial defects are associated with alterations in redox reactions, leading to the higher production of reactive oxygen species (ROS) (Turrens, 2003). ROS are detrimental to biological structures, and cells contain a large range of protective mechanisms against them (DiGiuseppe and Fridovich, 1984). The progressive collapse of these defenses has been postulated to be the main cause of aging, but there is no definitive evidence supporting this theory (Wickens, 2001). In their study, Chen and collaborators demonstrated for the first time that the respiratory complex I inhibitor rotenone, and respiratory complex II inhibitor thenoyl trifluoroacetone (TTFA) can induce autophagy and contribute to cell death in transformed and cancer cell lines (Chen et al., 2007). It has also been shown that ROS not only modulate autophagy (Kiššová et al., 2006) but further act as signaling molecules to activate autophagy in mammalian cells (Scherz-Shouval et al., 2007). These findings support the view that ROS-stimulated mitophagy might play a crucial role in the maintenance of healthy mitochondria by favoring the removal of damaged and free-radical-generating mitochondria. Other perturbations in the cellular environment can lead to the induction of autophagy and/or mitophagy, as is the case for insect cell lines responding to ATP depletion (Tettamanti et al., 2006), or coenzyme-Q-deficient fibroblasts (Rodriguez-Hernández et al., 2009).

In the present work, we have investigated autophagy induced by non-lethal concentrations of inhibitors of the mitochondrial respiratory chain: antimycin A and myxothiazol, that act on the same respiratory complex (ubiquinol–cytochrome c oxidoreductase, or complex III) but with distinct modes, leading to different levels of ROS production as well as an inhibitor of complex IV of the respiratory chain, potassium cyanide (KCN). When added to Saccharomyces cerevisiae grown under strict respiratory conditions, antimycin A and KCN, and to a lesser extent myxothiazol, induced autophagy and infrequent mitophagy. Furthermore, we report that certain components required for mitophagy are necessary for the induction of autophagy by these inhibitors, providing evidence for a strong connection between mitochondria status and autophagy. The level of reduced cytochrome b, one of the respiratory chain
components, could be the first sign in the pathway leading to the induction of autophagy in this setting.

**Results**

**Effects of antimycin A and myxothiazol on mitochondrial functions**

A large number of oxygen-dependent metabolic pathways are able to produce ROS, but the majority is formed as a byproduct of mitochondrial respiration, which represents about 90% of cellular oxygen consumption. The mitochondrial respiratory chain contains two main sites of production of superoxide ion from molecular oxygen, complexes I and III. Complex III (ubiquinol–cytochrome c oxidoreductase) is universally conserved in all respiratory chains, whereas complex I is absent from *S. cerevisiae* mitochondria. The increase of superoxide ion production in complex III occurs when the concentration of semi-ubiquinone increases. This happens with the classical respiratory chain inhibitor antimycin A, which blocks the reduction of semi-ubiquinone by cytochrome *b* (Fig. 1A) (Han et al., 2001). In contrast, myxothiazol, which blocks the oxidation of ubiquinone by iron-sulfur proteins, leads to the accumulation of this fully reduced component that does not react with molecular oxygen (Fig. 1A). In our study, antimycin A and myxothiazol were added to wild-type yeast cells growing in a lactate-supplemented medium. This non-fermentable carbon source is oxidized to pyruvate by two mitochondrial lactate dehydrogenases (de Vries and Marres, 1987), and a fully functional mitochondrial respiratory chain is required for its utilization. Antimycin A and myxothiazol were used at concentrations inducing a partial decrease of the respiration rate (Fig. 1B). Both compounds fully inhibited cell growth under these conditions (Fig. 1C). ROS production was measured by the conversion of dihydroethidium to fluorescent ethidium, and, as expected from their respective targets, antimycin A induced an increase in ROS production whereas myxothiazol did not (Fig. 1D). It should be noted that survival of cells treated with antimycin A (supplementary material Fig. S1) or myxothiazol (not shown) was not significantly affected.

**Antimycin A treatment does not induce mitophagy**

Since mitophagy is supposed to play a role in the degradation of unhealthy mitochondria as a housekeeping process in normal or stressed conditions, the potential impact of antimycin A treatment on this process was analyzed. Under growth conditions, lactate-grown cells exhibited a well-differentiated mitochondrial network. In order to follow the behavior of mitochondria in cells treated with inhibitor in vivo, two strains expressing GFP-tagged proteins that are targeted to the mitochondrial matrix were used: the first one bearing a construct expressing GFP fused downstream of the targeting sequence of Cit1 (mitochondrial citrate synthase) and the second one corresponding to a strain expressing the chimera Idp1–GFP (containing mitochondrial isocitrate dehydrogenase). Fluorescence microscopy showed that antimycin A treatment led to the partial fission of the mitochondrial network (Fig. 2A), and following this treatment cells appeared smaller in size. No mitochondrial GFP

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**Fig. 1. Effects of antimycin A and myxothiazol on a wild-type strain.** (A) Schematic representation of the action of antimycin A and myxothiazol on respiratory complex III. (B–D) Effect of antimycin A (2 μg/ml) and myxothiazol (3 μM) on cellular oxygen consumption (B), cell growth (C) and ROS level (D). Wild-type (W303-1B) cells were grown aerobically at 28°C in a lactate-supplemented YNB medium. (B) Oxygen consumption was measured at time 0 (control) or after addition of antimycin A (anti A) or myxothiazol (myxo). (C,D) Antimycin A or myxothiazol were added at time 0 and aliquots of the cultures were taken at the indicated times for cell growth and ROS measurements (see Materials and Methods). (D) Data are presented as the percentage of cells having a fluorescence above 10 units in the FL3 channel. Each measurement corresponds to the mean ± s.d. of at least three experiments.
fluorescence was detected inside vacuoles under these conditions suggesting that altered mitochondria may not be a target for degradation by mitophagy.

To extend the analysis of mitophagy, we examined possible vacuolar degradation of mitochondria by western blot analysis. Upon delivery of GFP-tagged proteins to the vacuole, part of the chimera is typically cleaved and/or degraded, but the GFP moiety, being more resistant to hydrolases, accumulates in the vacuolar lumen (Shintani and Klionsky, 2004). Thus, the accumulation of free GFP can be used to monitor the extent of autophagy. Furthermore, the type of autophagy can be determined based on the nature of the chimera. For example, GFP–Atg8 is used to monitor nonspecific autophagy (although this hybrid can also be degraded during selective autophagy) in response to starvation, whereas organelle-specific constructs such as Pex14–GFP (Shintani and Klionsky, 2004) can be used to follow peroxisome-specific, or other types of selective autophagy. Accordingly, we utilized the two different mitochondrially targeted chimeras containing Cit1 or Idp1 fused to GFP, which are markers of mitophagy. As expected, nitrogen starvation induced substantial degradation of mitochondria as seen by the appearance of free GFP from either construct (Fig. 2B,C, ‘-N’). In contrast, there was no indication of mitophagy in response to antimycin A treatment (Fig. 2B,C).

The apparent lack of induction of mitophagy following treatment with antimycin A compared to starvation suggests a fundamentally different cellular response to mitochondrial damage, at least for cells that are growing under strict respiratory conditions. To further support this conclusion, we carried out electron microscopy (EM) to monitor mitophagy under our experimental conditions. Cells were grown in medium containing lactate (or starved as a control), treated with antimycin A or myxothiazol, and processed for EM analysis as described in Materials and Methods. When cells were starved for 2 or 3 hours, we detected substantial numbers of cells with mitochondria present in the vacuole lumen, and this increased in a time-dependent manner with ~85% of the cells displaying this phenotype at the later time point (supplementary material Table S1). In contrast, very few cells displayed mitochondria within the vacuole (~7%), even after 14 or 24 hours of treatment with antimycin A suggesting that these conditions lead to degradation of mitochondria rarely, and hence it was not recorded by fluorescence microscopy or western-blot experiments (Fig. 3A–D; supplementary material Table S1). The lack of any increase in

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**Fig. 2. Effect of antimycin A on mitophagy.** (A) Wild-type (W303-1B) cells expressing Cit1–GFP or Idp1–GFP were grown in a lactate-supplemented YNB medium. Antimycin A (2 μg/ml) was added at time 0. Cells were observed by fluorescence microscopy at time 0 and after 14 hours of incubation with antimycin A. Cells expressing Cit1–GFP are shown, but essentially identical results were obtained for cells expressing Idp1–GFP. (B,C) At the indicated times, aliquots of cells from strains expressing (B) Cit1–GFP or (C) Idp1–GFP were harvested and total extracts were analyzed on western blots probed with antibodies against GFP and Pgk1 (the latter as a loading control). Lane -N shows extracts from starved cells.

**Fig. 3. Antimycin-A-induced mitophagy and macroautophagy.** Electron microscopy analysis of the wild-type (W303-1B) strain grown in a lactate-supplemented YNB medium and treated for 24 hours with (A–D) antimycin A (2 μg/ml) or (E,F) myxothiazol (3 μM). Mitophagy is demonstrated by the presence of mitochondria within vacuoles (m). Macroautophagy is indicated by the presence of autophagosomes (A) and autophagic bodies (AB). Rare autophagosomes may eventually contain one mitochondrion. N, nucleus; V, vacuole.
mitochondrial delivery to the vacuole between these time points suggests that this is not a specific degradation of mitochondria, but rather a very low level of nonspecific degradation. Along these lines, we also monitored the formation of nonspecific autophagosomes. Although starvation induces autophagy, most cells do not contain detectable autophagosomes under these conditions, presumably reflecting efficient autophagic flux (supplementary material Table S1). In contrast, cells treated with antimycin A displayed a substantial number of autophagosomes, and again this number increased in a time-dependent manner. These findings suggest that antimycin A induces autophagosome formation, although the autophagic flux may not be able to keep pace with the generation of autophagosomes. Myxothiazol treatment did not result in the detection of any mitochondria within the vacuole, and had a very minor effect with regard to autophagosome accumulation (Fig. 3E–F; supplementary material Table S1).

**Antimycin A treatment induces nonspecific autophagy**

Having established that antimycin A did not induce mitophagy, we turned to an examination of nonselective autophagy. Cells were grown in lactate-containing medium, treated with antimycin A or myxothiazol, and examined for nonspecific autophagy using extracts from starved cells. Although starvation induces autophagy, most cells do not contain detectable autophagosomes under these conditions, presumably reflecting efficient autophagic flux (supplementary material Table S1). In contrast, cells treated with antimycin A displayed a substantial number of autophagosomes, and again this number increased in a time-dependent manner. These findings suggest that antimycin A induces autophagosome formation, although the autophagic flux may not be able to keep pace with the generation of autophagosomes. Myxothiazol treatment did not result in the detection of any mitochondria within the vacuole, and had a very minor effect with regard to autophagosome accumulation (Fig. 3E–F; supplementary material Table S1).

**Antimycin A treatment induces nonspecific autophagy**

Having established that antimycin A did not induce mitophagy, we turned to an examination of nonselective autophagy. Cells were grown in lactate-containing medium, treated with antimycin A or myxothiazol, and examined for nonspecific autophagy using both the Pho8Δ60-dependent alkaline phosphatase (Noda et al., 1995) and GFP–Atg8 processing assays. When cells were subjected to nitrogen starvation there was a substantial increase in nonspecific autophagy as expected (Fig. 4A; compare time 0 to ‘-N’). Over the time course of the experiment (24 hours), a weak stimulation of basal autophagic activity was observed in wild-type (W303-1B) cells (Fig. 4A, white bars), that may correspond to the housekeeping recycling of altered biological material in cells approaching the stationary phase of growth, visualized also on western blots by following GFP–Atg8 processing (Fig. 4B, control). Myxothiazol did not induce a stimulation of autophagy above this basal level (Fig. 4A, hatched bars). In contrast, antimycin A markedly stimulated autophagic activity (Fig. 4A, black bars). The induction of a nonspecific autophagic process by antimycin A treatment was also confirmed by following GFP–Atg8 processing by western blot. In wild-type (W303-1B) cells, the appearance of a free GFP band was already detected by 2 to 4 hours after antimycin A addition, and it reached a maximum level after 8 hours, revealing the induction of an autophagic process (Fig. 4B). The same results were obtained for another wild-type strain (BY4742; Fig. 4C). Again, myxothiazol treatment induced a lower, although in this case detectable, autophagic response than did antimycin A (Fig. 4D), in agreement with the Pho8Δ60 (Fig. 4A) and EM (Fig. 3E–F) analyses.

Antimycin A and myxothiazol treatments lead to mitochondrial dysfunction, the former being associated with a massive production of ROS. To test the possible role of ROS in the effect of antimycin A, antioxidants were added to cells treated with this drug, but this did not inhibit autophagy induction (supplementary material Fig. S2A–B). Conversely, we observed that the treatment of cells with the strong oxidant H2O2 did not induce autophagy (supplementary material Fig. S2C). These results suggest that ROS production may have contributed to autophagy induction following treatment with antimycin A, but that oxidative stress is not essential for this response, and is not able to induce this response by itself.

ROS being excluded and in order to explore more in detail how autophagy could be induced following mitochondrial alterations, we examined also the effect of other inhibitors of mitochondrial oxidative phosphorylation such as KCN (an inhibitor of cytochrome oxidase), oligomycin (an inhibitor of the F0–F1 ATP synthase) and CCCP (carbonyl cyanide m-chlorophenyl hydrazone, a decoupling agent) (Fig. 5A). Treatment with CCCP and oligomycin did not induce autophagy despite the fact that with CCCP a slight band appeared after 24 hours of treatment. In contrast, the use of KCN caused an appreciable level of autophagy, like in the case of antimycin A (Fig. 5A; supplementary material Fig. S3). The simultaneous addition of antimycin A and KCN to the cells induced autophagy at about the same level as in the case of the addition of each drug separately (Fig. 5A; supplementary material Fig. S3) suggesting that the effect of antimycin A and KCN is not cumulative.

Furthermore, cells were treated with different combinations of drugs: KCN+H2O2, antimycin A+H2O2 or myxothiazol+H2O2. In
these three conditions, autophagy was not induced (Fig. 5B). Interestingly, these results may be explained by the fact that depending on the inhibitor used, some components of the respiratory chain were reduced or oxidized. In fact, it was previously shown that antimycin A treatment leads to a higher cytochrome b reduction than myxothiazol (von Jagow and Engel, 1981). We have confirmed these data by measuring cytochrome b reduction after addition of antimycin A, myxothiazol, KCN or KCN in combination with antimycin A on isolated mitochondria (Fig. 5C). Results obtained with the different inhibitors showed that only when cytochrome b is reduced, is autophagy induced and consequently this high level of reduced cytochrome b could be the first mitochondrial signal involved in this type of autophagy induction.

**Autophagy induction by antimycin A requires the canonical autophagy-related proteins**

Several reports have indicated that autophagy can be carried out by noncanonical pathways that do not require some of the autophagy-related (Atg) proteins (for a review, see Codogno et al., 2011). To further explore the mechanism of antimycin-A-induced autophagy we examined the role of Atg proteins in this process. Atg1 and Atg13 are two proteins that play a crucial role in the initiation of autophagy. Tor is a serine/threonine kinase, and its activity is inhibited by nutrient starvation or rapamycin treatment, which has been established as a crucial step for autophagy induction in eukaryotes (Noda and Ohsumi, 1998; Kamada et al., 2000). During growth conditions, Tor is active, and Atg13 is hyperphosphorylated. Upon a shift to starvation conditions, or following rapamycin treatment, Atg13 is rapidly dephosphorylated, and this alteration correlates with the induction of autophagy. The dephosphorylation of Atg13 can be conveniently monitored as an increase in its electrophoretic mobility following SDS-PAGE (Scott et al., 2000). Antimycin A treatment resulted in an increase in the mobility of Atg13 indicative of dephosphorylation similar to that seen with starvation (not shown). This result suggested that autophagy induction by antimycin A occurred through TOR-dependent events that are correlated with Atg13 dephosphorylation.

Next, we examined the role of different Atg proteins in antimycin-A-induced autophagy. Atg1, Atg9, Atg13 and Atg17 proteins are considered to be part of the core machinery (Xie and Klionsky, 2007) required for all types of autophagy, and strains having deletions in any of the corresponding genes displayed complete blocks in antimycin-A-induced autophagy (Fig. 6). The atg29Δ and atg31Δ mutants are not required for the Cvt pathway, display partial defects in nonspecific autophagy, and are required for mitophagy (having ~100% and 50% blocks in the latter, respectively (Kawamata et al., 2005; Kabeya et al., 2007). Similar to the atg1Δ, atg9Δ, atg13Δ and atg17Δ mutants, strains deficient in Atg29 or Atg31 showed an essentially complete block in antimycin-A-induced autophagy. In contrast, a deletion of ATG19 had no effect on this process, which is in agreement with the role of Atg19 as a cytoplasm-to-vacuole targeting
pathway-specific component (Shintani and Klionsky, 2004). These results indicate that the canonical autophagy machinery is required for antimycin-A-induced autophagy.

Components of mitophagy are required to induce autophagy

In mammalian cells it is established that mitophagy contributes to the quality control process needed to maintain a functional pool of mitochondria; however, specific molecular components that are involved in mitophagy have not been well characterized. In contrast, there is a better understanding of the regulation of mitophagy in yeast. Recent data defined two categories of yeast mitophagy components: those constitutively required for mitophagy such as Atg32 and Atg11, and those having a regulatory function in mitophagy such as Ptc6/Aup1 and Atg33. Genomic screens have also identified other proteins that are partially required for mitophagy (Okamoto et al., 2009; Kanki et al., 2009a; Kanki et al., 2009b). As a representative of the latter group we chose dnm1Δ in this study. In order to follow the involvement of these proteins in the regulation of autophagy induction under the particular conditions used in our study, strains individually lacking each of these proteins were treated with antimycin A.

Respiratory activities in all strains were inhibited by the addition of antimycin A to the same extent as in wild-type cells (50% inhibition with 0.4 μg antimycin A/ml; data not shown) and autophagy induction was examined. Based on the GFP–Atg8 processing assay, there was a severe block in autophagy in the atg11Δ and atg32Δ strains (Fig. 7) even though these mutants display no defect in nonspecific autophagy under starvation conditions (Okamoto et al., 2009; Kanki et al., 2009b). As a representative of the latter group we chose dnm1Δ in this study. In order to follow the involvement of these proteins in the regulation of autophagy induction under the particular conditions used in our study, strains individually lacking each of these proteins were treated with antimycin A.

Respiratory activities in all strains were inhibited by the addition of antimycin A to the same extent as in wild-type cells (50% inhibition with 0.4 μg antimycin A/ml; data not shown) and autophagy induction was examined. Based on the GFP–Atg8 processing assay, there was a severe block in autophagy in the atg11Δ and atg32Δ strains (Fig. 7) even though these mutants display no defect in nonspecific autophagy under starvation conditions (Okamoto et al., 2009; Kanki et al., 2009b). Similarly, EM observations showed no indication of autophagy in the atg32Δ strain (Fig. 8). In contrast, antimycin A treatment induced autophagy in ptc6Δ, atg33Δ and dnm1Δ strains at a level similar to wild type (Fig. 7; supplementary material Fig. S4).

The Rtg pathway is induced by antimycin A but is not required for antimycin A-induced autophagy

The retrograde (Rtg) response is an intracellular signaling pathway that signals mitochondrial dysfunction, or more specifically electron transport chain disruption, to the nucleus causing wide-ranging adaptations to the resultant metabolic stress (Butow and Avadhani, 2004). The consecutive changes in nuclear gene expression lead to the synthesis of enzymes that compensate for the mitochondrial dysfunction. The prototypical target gene of the retrograde pathway is CIT2, which encodes a peroxisomal isoform of citrate synthase (Liao et al., 1991). In cells compromised in mitochondrial functions, for example, those without mtDNA (rho0 petites), CIT2 expression is increased by as much as 30-fold (Liao et al., 1991). We followed the induction of the Rtg pathway in different strains treated with antimycin A by monitoring Cit2–GFP by western blot (supplementary material Fig. S5A). The data indicated that the Rtg pathway was functional and induced by antimycin A in all the strains tested including atg32Δ and ptc6Δ//aup1Δ indicating that a defect in this pathway was not the cause of nonspecific autophagy. However, antimycin A treatment of mutants that are defective in the Rtg pathway, rtg1Δ and mks1Δ, induced autophagy, suggesting that the Rtg response is not needed for nonspecific autophagy induction under these conditions (supplementary material Fig. S5B).

Bck1 kinase is required for autophagy induction following antimycin A treatment

Considering that the Rtg pathway is not required for antimycin-A-induced autophagy, we decided to examine different signaling pathways that have been characterized as playing a role in the regulation of autophagy (Budovskaya et al., 2004; Yorimitsu et al., 2007). The Ras–cAMP-dependent protein kinase A (PKA) signaling pathway is the primary glucose sensor that functions as a negative regulator of autophagy, controlling many aspects of cell growth in response to extracellular nutrients and stress conditions (Thevelein and de Winde, 1999). Moreover, data confirm that the mitochondrial content adjustment that occurs during growth is related to the activity of this pathway. Specifically, in the transition phase, yeast Tpk3, one of the catalytic subunits of PKA, plays a major role in this process (Chevtzoff et al., 2005. In addition to these kinases, data also underscore the importance of Sch9 in cellular signaling (Fabrizio
Moreover, recent data show that two MAPK-signaling pathways (including Slt2, Hog1 and Bck1) are required for the regulation of mitophagy (Mao et al., 2011). We investigated the behavior of different kinase mutants following antimycin A treatment. In these conditions, autophagy was induced in kinase mutants defective for Tpk3 or Sch9 at levels similar to the wild type. In the hog1Δ mutant, expression of GFP–Atg8 was low, but autophagy was still induced by antimycin A treatment. In contrast, in the bck1Δ mutant and to a lesser extent in the slt2Δ mutant, induction of autophagy was very low (Fig. 9; supplementary material Fig. S6). These results showed that the bck1Δ mutant and to a lesser extent slt2Δ mutant had the same behavior as mitophagy-deficient mutants such as atg32Δ, or atg11Δ.

Discussion

Recently, the concept of selective mitochondria autophagy has been documented by a growing number of reports. It has been hypothesized that selective autophagy of mitochondria (mitophagy) might act as a protective process against the accumulation of damaged organelles, that may come from oxidative stress, ageing, mitochondrial pathologies or aborted apoptosis; thus, mitophagy may act as one of the cellular strategies involved in the mitochondrial quality control process, acting to remove dysfunctional organelles. Accordingly, the inhibition of the respiratory chain by the selective inhibitors antimycin A and myxothiazol provided a useful method to test this hypothesis. These drugs act on the same respiratory complex, albeit with distinct mechanisms, leading to similar decreases in respiration rates but different levels of ROS production. Contrary to our predictions, antimycin A and myxothiazol did not induce massive mitophagy. Instead, antimycin A triggered nonspecific autophagy.

Previous reports have already suggested that mitochondria dysfunction can induce autophagy. For example, in yeast, mutations in ATP synthase biogenesis (fmc1Δ) or the inactivation of the K+/H+ antiporter (mdm38Δ) induce autophagic processes that include mitochondrial degradation (Priault et al., 2005; Nowikovsky et al., 2007). Tettamanti et al. showed that intracellular ATP depletion of the IPLB-LdFB insect line by oligomycin triggers an autophagic process that mainly targets mitochondria (Tettamanti et al., 2006). All these reports described extreme conditions leading to dramatic mitochondrial defects. In our study, mitochondrial alterations caused by antimycin A or myxothiazol were moderate, since respiration was not completely inhibited. Interestingly, it was recently shown that in human cells antimycin A can inhibit basal, rapamycin-induced and serum-starvation induced autophagy independently.
of ATP and ROS (Ma et al., 2011). Surprisingly, in these cells antimycin A did not have an obvious effect on ROS production. They propose that complex III, the target of antimycin A, can regulate a signaling event of autophagy through the HIF1A-BNIP3 cascade or, alternatively, the block in autophagy may indicate the involvement of mitochondria as a source of autophagic membranes. At any rate, these data argue for significant differences in these two processes between yeast and mammalian cells. Such a difference is not unexpected considering that yeast preferentially carry out fermentation, whereas most human cells are restricted to aerobic metabolism.

There is often a strong correlation between mitochondria alteration and the increase of intracellular ROS levels (Turrens, 2003). Furthermore, increases in ROS might trigger autophagy (Kisšová et al., 2006; Scherz-Shouval et al., 2007; Kirkland et al., 2002; Kiffin et al., 2006). For example, the downregulation of catalase induces autophagy in mammalian cells (Yu et al., 2006). Oxidative stress induces autophagy in Arabidopsis seedlings in an AtATG18a-dependent manner, and transgenic lines that are defective in autophagosome formation are hypersensitive to ROS (Xiong et al., 2007). These results also showed that the absence of a functional autophagy pathway induces a constitutively high level of oxidative stress in plants grown under normal conditions (Xiong et al., 2007).

The present report supports the hypothesis that moderate mitochondria dysfunction is able to trigger autophagy. Moreover, the different outcomes obtained from treatment of cells with antimycin A, myxothiazol and KCN support the hypothesis that cumulative injuries are required to activate/induce the process: the inhibition of respiration by myxothiazol was not sufficient to trigger a detectable autophagic process, but the combination of respiration inhibition and ROS level increase by antimycin A or the inhibition of respiration by KCN allowed the stimulation of the autophagic process at a significant level. However, it was observed that a treatment of cells with the strong oxidant hydrogen peroxide (H$_2$O$_2$) did not induce autophagy, and that antioxidants did not affect autophagy activation caused by antimycin A or KCN. On the contrary, the combination of inhibitors antimycin A or KCN with H$_2$O$_2$ restrained the autophagy induction showing that an oxidized mitochondria state prevents this induction. All these results seem to indicate that increased levels of reduced cytochrome $b$ could be the first signal required for this induction. These results could be related to those of Ma et al. who have shown the involvement of complex III in autophagy regulation (Ma et al., 2011).

Interestingly, antimycin-A-induced autophagy was impaired in mitophagy-deficient mutants $atg11A$ and $atg32A$. While Atg11 is required for all types of selective autophagy, Atg32 is exclusively mitophagy specific. It is particularly striking that although this protein is not involved in nonselective autophagy induced by starvation it is required for antimycin-A-induced autophagy. In contrast, the absence of proteins involved in autophagy whose function is not essential for this process in general, such as Atg33, Ptc6/Aup1 and Dnm1, does not influence antimycin-A-induced autophagy. Thus, the present report suggests the possibility that some indispensable components of mitophagy participate in a signaling pathway required for autophagy induction following mitochondria dysfunction/alteration.

The retrograde signaling pathway is a mechanism that induces transcriptional responses to mitochondrial stress cues (Liu and Butow, 2006; Liao et al., 1991; Parikh et al., 1987). We found that the Rtg pathway was induced after antimycin A treatment, but was not required for autophagy induction. This shows that independent responses (i.e. the Rtg pathway and autophagy) can be induced to protect cells from mitochondrial injuries. It is known that several kinases can interfere with the mitochondrial status. Among those that have been tested in this study, only Bck1 and to a lower level Slt2 participate in the mitophagy-signaling pathway. It was recently shown that the MAPK Slt2 and upstream components including Bck1 are involved in mitophagy regulation (Mao et al., 2011).

Several lines of data support the emerging concept of an alternative role for autophagy as a stress-induced housekeeping mechanism involved in the general maintenance of cellular homeostasis and in particular the quality control of mitochondria (Lemasters, 2005; Levine and Klionsky, 2004; Mijaljica et al., 2007). A previous study by Zhang et al. clearly indicates that a
defect in any of several ATG genes (ATG1, ATG6, ATG8 and ATG12) has an impact on various aspects of mitochondrial function, suggesting a critical role of autophagy in mitochondria maintenance (Zhang et al., 2007). More recently, Ohsumi’s laboratory has established an important role for autophagy in maintaining mitochondrial function by supporting essential protein synthesis. They showed that mitochondria dysfunction using rho0 mutants, demonstrated that a regulatory link exists between mitochondrial function and autophagy during nitrogen starvation (Graef and Nunnari, 2011). In their study, these authors showed that mitochondrial respiratory deficiency (i.e. the rho0 mutant) suppresses autophagy induction and autophagy flux, both dependent on PKA activity. In our study, the situation is very different since wild-type and mutant cells were grown on a strictly respiratory carbon source, and were treated with low doses of respiratory chain inhibitors leading to only a partial inhibition of respiratory activity. Under these conditions, autophagy but not massive mitochondria autophagy, was induced. Taken together, these data strongly support the view that autophagy participates in mitochondrial quality control.

Depending on experimental conditions, we propose that moderate mitochondrial dysfunction can directly contribute to a signaling pathway leading to the induction of autophagy (Fig. 10). In this report, we identified the first signal and some of the proteins involved in this pathway, including Atg32. However, further studies will be needed to provide details about the nature of this process, and to improve our understanding of the role of autophagy in the quality control of mitochondria.

Materials and Methods

Yeast strains, plasmids and growth conditions

The yeast strains used in this study are listed in supplementary material Table S2. W303-1B (MATa, ade2, his3, leu2, trpl, ura3, can1) and BY4742 (Euroscarf) were used as wild-type strains. The atg3A and sch9A strains were made in the W303-1B background. Strains atg11A, atg22A, ptc6A, dim1A, rgl1A, msk1A, tor1A, tpk3A, scd6A, bck2A, hoc1G, scd2A, ang1A, ang4A, atg13A, atg7A, atg19A, atg29A, and atg31A were obtained from Euroscarf. For the measurement of Pho860-dependent alkaline phosphatase activity, the PHO8 locus was replaced with PHO8::600 in each strain by transformation with a HindIII fragment of the plasmid pTN9 (a gift from Dr Yoshinori Ohsumi, Tokyo Institute of Technology, Japan) bearing PHO8::600 as described previously (Kiššová et al., 2004). For GFP-Atg8 expressing, the plasmid pRS416 expressing GFP-Atg8 was used (Noda et al., 1995). A plasmid expressing Idp1–GFP was a gift from Dr Haga Abeliovich (Hebrew University of Jerusalem, Israel). The Cat2–GFP strain was a gift from Dr Benoit Pinson (IBGC, Bordeaux, France). Yeast cells were grown aerobically at 28°C in a minimal medium, YNB [0.175% yeast nitrogen base with or without amino acids and ammonium sulfate, 0.5% ammonium sulfite, 0.1% potassium phosphate, 0.2% Drop-Mix (mixture of amino acids except auxotrophic requirements), 0.01% auxotrophic requirements, pH 5.5] supplemented with 2% lactate as a carbon source. Growth of cells was followed by measurement of optical density (OD) at 600 nm. In some experiments, starved cells (for 5 hours) were used as a positive control and prepared as previously described (Kiššová et al., 2004).

Plating Efficiency

The viability of cells was determined as plating efficiency. The cells were counted at indicates times and diluted, and aliquots with identical numbers of cells (5 × 10^6) were plated on solid completeYPD (1% yeast extract, 2% peptone, and 2% dextrose) or YPL (1% yeast extract, 2% peptone, and 2% lactate) medium, respectively. The number of colonies was scored after 2–3 days of growth at 28°C. The viability of cells in each sample was expressed as the percentage of colonies produced by the plating of the same aliquots of culture at time 0.

ROS measurement

Aliquots of cells (5 × 10^6 cells/ml) were incubated for 15 min with agitation in the presence of 20 μM dihydroethidium (Sigma, D7003). Following oxidation by ROS, dihydroethidium is converted to ethidium. Fluorescence was measured in a Galaxy flow cytometer (Partec). When used, antimycin A (2 μg/ml; Sigma, A8674) and myxothiazol (3 μM; Sigma, M5779) were added in the growth medium.

Cellular respiration

Respiration rates were measured at 28°C with a Clark-type electrode in a 1 ml chamber at 28°C. Cells were diluted in a fresh growth medium and respiration rates were determined from the slope of O2 concentration versus time.

Measurement of cytochrome reduction

Mitochondria were isolated according to Guérin et al. (Guérin et al., 1979). Cytochrome reduction was measured by redox spectrophotometry. Briefly, mitochondria (4 mg proteins/ml) were suspended in the two cuvettes of a double-beam spectrophotometer (Varian Cary 4000). The reference cuvette was oxidized with H2O2 and the sample cuvette was reduced with ethanol + inhibitor. The cytochrome b spectrum was acquired between 561 and 575 nm.

Alkaline phosphatase assay

The alkaline phosphatase activity assay using β-naphthyl phosphate (Sigma, N7255) as a substrate was performed on cells that had been treated without or with antimycin A or myxothiazol according to the method described previously (Kiššová et al., 2004). Fluorescence intensity was measured at 472 nm (excitation at 345 nm) in a Safas Xenius spectrofluorimeter. Protein concentration was measured with the Lowry method (Lowry et al., 1951). ALP activities were expressed as arbitrary fluorescence units/minute/mg proteins (AU/min/mg).

Epifluorescence microscopy

To induce mitochondrial apoptosis, cells carrying the GAL-p-CLBGP (a gift from Dr Jean-Paul Di Rago, IBGC, Bordeaux, France) plasmid were grown in the appropriate medium supplemented with 0.5% galactose. GFP was visualized on an epifluorescence microscope (Leica Microsystems DM-ILB). The images were acquired with a SIS camera and processed with Corel draw 9.1 suite software.

Immunoblotting of mt–GFP, GFP–Atg8, Pgk1 and Cit2–GFP

To monitor autophagy, whole-cell extracts of approximately 2 × 10^7 cells were harvested, resuspended in 0.5 ml of water, and added to 50 μl of a mixture of 3.5% β-mercaptoethanol in 2 M NaOH in a 1.7-ml microcentrifuge tube. After a 15-min incubation on ice, proteins were precipitated by adding 50 μl of 3 M trichloroacetic acid for 15 min on ice. After centrifugation for 10 min at 10,000 g, the pellet fraction was washed with 0.2 ml of acetone, dried and resolubilized in 20 μl of 5% SDS plus 20 μl Laemmli buffer (2% β-mercaptoethanol, 2% SDS, 0.1 M Tris-HCl, pH 8.8, 20% glycerol, 0.02% Bromophenol Blue). Samples were incubated at 70°C for 5 min prior to loading on gels. Cells lysates equivalent of 0.2 × 10^7 cells were subjected to immunoblot analysis with anti-GFP antibody (Roche, 1:5000 dilution), or with anti-Pgk1 antibody (Invitrogen, 1:5000 dilution). Mouse secondary antibodies were used at a dilution of 1:5000. Detection was done with ECL+ reagent from Amersham.

Electron microscopy

For electron microscopy experiments, cells were grown in the presence of 1 mM PMSF. Harvested cells were placed on the surface of Formvar-coated copper grids (400 mesh). Each loop was quickly submersed in liquid propane (~190°C) and then transferred to a precooled solution of 4% osmium tetroxide in dry acetone at ~82°C for 48 h for substitution/fixation. Samples were gradually warmed to room temperature, and washed in dry acetone. Specimens were stained for 1 h with 1% uranyl acetate in acetone at 4°C, rinsed and infiltrated with araldite (epoxy resin, Fluka; ref: 10951-1L). Ultra-thin sections were stained with lead citrate. Observations were performed on a Philips Tecnai 12 Biotwin (120kV) electron microscope.

Acknowledgements

The authors wish to thank Cyrille Chevtzoff (CNRS UMR5095) for help with respiration measurements; Dr Yoshinori Ohsumi and Dr Hagai Abeliovich for the gift of plasmids or strains; and Drs Muriel Priault, Anne Devin and Michel Rigoulet (CNRS UMR5095) for helpful discussions.

Author contributions

M.D. and I.B.-K. performed the majority of experiments; B.S. performed electron microscopy experiments; D.J.K. performed experiments and participated in the improvement of the manuscript; B.P. constructed mutants strains; N.C. designed the project, performed experiments and analysed data with I.B.-K.; N.C., I.B.-K., S.M. wrote the manuscript with comments from co-authors.
Mitochondrial alterations and autophagy

Funding
This work was supported by grants from CNRS [UMR5095], the Université Victor Segalén [UMR5095]; from the Association Française contre les myopathies [grant number 14644 to N.C.]; from the Slovak Agency VEGA [grant number 1/0264/08 to I.B.-K.]; a grant G5M3393 to D.J.K.; a post-doc fellowship from the Fondation pour la Recherche Medicale to I.B.-K. and a fellowship from the Ligue Nationale contre le Cancer to M.D. France-Slovakia collaboration was supported by Egide [Project EcoNet number 101877V to S.M.].

Supplementary material available online at http://jcs.biologists.orglookup/suppl/doi:10.1242/jcs.103713/-/DC1

References


Fig. S1. Viability of wild-type strains after 24 hours of antimycin A treatment. Wild-type W303-1B and BY4742 cells expressing GFP-Atg8 were grown in a lactate-supplemented YNB medium. After 24 hours of antimycin A treatment (2 µg/ml), viability of cells was measured as described in Material and Methods.

Fig. S2. Effect of antioxidants on autophagy induction. (A) Wild-type (W303-1B) cells expressing GFP-Atg8 were grown in a lactate-supplemented YNB medium. At different optical densities, aliquots of cells were harvested and total extracts were analyzed by western blot. (A). At time 0, antimycin A (2 µg/ml) was added in the absence or presence of N-acetyl cysteine (NAC, 5 mM) or resveratrol (Resv, 100 µM). (B) ALP activities were measured in wild-type (W303-1B) cells incubated with antimycin A (2 µg/ml) in the presence or absence of NAC (5 mM), or resveratrol (100 µM) resp. for 14 hours. (C) At time 0, H2O2 (5 mM) was added and at the indicated times, aliquots of cells were harvested and total extracts were analyzed by western blot.
**Fig. S3. Quantification of western blots** of the GFP-Atg8 processing assay by Image J program: the graph shows the ratio GFP over GFPAtg8 + GFP after 14 hours of treatment of wild-type cells with antimycin A, KCN or a combination of antimycin A and KCN.

**Fig. S4. Effect of antimycin A on the dnm1Δ mutant strain.** Mutant dnm1Δ cells expressing GFP-Atg8 were grown in a lactate-supplemented YNB medium. At time 0, antimycin A (2 µg/ml) was added and at the indicated times, aliquots of cells were harvested and total extracts were analyzed by western blot.
Fig. S5. Induction of the Rtg pathway following antimycin A treatment. (A) Wild-type (W303-1B) and mitophagy mutant cells expressing Cit2-GFP or (B) Rtg pathway mutants were grown in a lactate-supplemented YNB medium. Antimycin A (2 µg/ml) was added at time 0. At the indicated times, aliquots of cells were harvested and total extracts were analyzed by western blot.

Fig. S6. Quantification of western blots of the GFP-Atg8 processing assay by ImageJ program: the graph shows the ratio of GFP over GFP-Atg8 + GFP after 8 hours of antimycin A treatment.
Table S1. Quantification of structures observed by electron microscopy

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Cells were treated as detailed in Materials and Methods. *From Kiššová et al., 2007
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