Glutathione Participates in the Regulation of Mitophagy in Yeast*

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The antioxidant \( \text{N-acetyl-L-cysteine} \) prevented the autophagy-dependent delivery of mitochondria to the vacuoles, as examined by fluorescence microscopy of mitochondria-targeted green fluorescent protein, transmission electron microscopy, and Western blot analysis of mitochondrial proteins. The effect of \( \text{N-acetyl-L-cysteine} \) was specific to mitochondrial autophagy (mitophagy). Indeed, autophagy-dependent activation of alkaline phosphatase and the presence of hallmarks of non-selective microautophagy were not altered by \( \text{N-acetyl-L-cysteine} \). The effect of \( \text{N-acetyl-L-cysteine} \) was not related to its scavenging properties, but rather to its fueling effect of the glutathione pool. As a matter of fact, the decrease of the glutathione pool induced by chemical or genetical manipulation did stimulate mitophagy but not general autophagy. Conversely, the addition of a cell-permeable form of glutathione inhibited mitophagy. Inhibition of glutathione synthesis had no effect in the strain \( \Delta \text{uth1} \), which is deficient in selective mitochondrial degradation. These data show that mitophagy can be regulated independently of general autophagy, and that its implementation may depend on the cellular redox status.

Autophagy is a major pathway for the lysosomal/vacuolar delivery of long-lived proteins and organelles, where they are degraded and recycled. Autophagy plays a crucial role in differentiation and cellular response to stress and is conserved in eukaryotic cells from yeast to mammals (1, 2). The main form of autophagy, macroautophagy, involves the non-selective sequestration of large portions of the cytoplasm into double-membrane structures termed autophagosomes, and their delivery to the vacuole/lysosome for degradation. Another process, microautophagy, involves the direct sequestration of parts of the cytoplasm by vacuole/lysosomes. The two processes co-exist in yeast cells but their extent may depend on different factors including metabolic state: for example, we have observed that nitrogen-starved lactate-grown yeast cells develop microautophagy, whereas nitrogen-starved glucose-grown cells preferentially develop macroautophagy (3).

Both macroautophagy and microautophagy are essentially non-selective, in the way that autophagosomes and vacuole invaginations do not appear to discriminate the sequestered material. However, selective forms of autophagy have been observed (4) that target namely peroxisomes (5, 6), chromatin (7, 8), endoplasmic reticulum (9), ribosomes (10), and mitochondria (3, 11–13). Although non-selective autophagy plays an essential role in survival by nitrogen starvation, by providing amino acids to the cell, selective autophagy is more likely to have a function in the maintenance of cellular structures, both under normal conditions as a “housecleaning” process, and under stress conditions by eliminating altered organelles and macromolecular structures (14–16). Selective autophagy targeting mitochondria, termed mitophagy, may be particularly relevant to stress conditions. The mitochondrial respiratory chain is both the main site and target of ROS\(^4\) production (17). Consequently, the maintenance of a pool of healthy mitochondria is a crucial challenge for the cells. The progressive accumulation of altered mitochondria (18) caused by the loss of efficiency of the maintenance process (degradation/biogenesis \( \text{de novo} \)) is often considered as a major cause of cellular aging (19–23). In mammalian cells, autophagic removal of mitochondria has been shown to be triggered following induction/blockade of apoptosis (23), suggesting that autophagy of mitochondria was required for cell survival following mitochondria injury (14). Consistent with this idea, a direct alteration of mitochondrial permeability properties has been shown to induce mitochondrial autophagy (13, 24, 25). Furthermore, inactivation of catalase induced the autophagic elimination of altered mitochondria (26). In the yeast \( \text{Saccharomyces cerevisiae} \), the alteration of \( F_{0}F_{1} \) ATPase biogenesis in a conditional mutant has been observed (4) that target namely peroxisomes (5, 6), chromatin (7, 8), endoplasmic reticulum (9), ribosomes (10), and mitochondria (3, 11–13). Although non-selective autophagy plays an essential role in survival by nitrogen starvation, by providing amino acids to the cell, selective autophagy is more likely to have a function in the maintenance of cellular structures, both under normal conditions as a “housecleaning” process, and under stress conditions by eliminating altered organelles and macromolecular structures (14–16). Selective autophagy targeting mitochondria, termed mitophagy, may be particularly relevant to stress conditions. The mitochondrial respiratory chain is both the main site and target of ROS\(^4\) production (17). Consequently, the maintenance of a pool of healthy mitochondria is a crucial challenge for the cells. The progressive accumulation of altered mitochondria (18) caused by the loss of efficiency of the maintenance process (degradation/biogenesis \( \text{de novo} \)) is often considered as a major cause of cellular aging (19–23). In mammalian cells, autophagic removal of mitochondria has been shown to be triggered following induction/blockade of apoptosis (23), suggesting that autophagy of mitochondria was required for cell survival following mitochondria injury (14). Consistent with this idea, a direct alteration of mitochondrial permeability properties has been shown to induce mitochondrial autophagy (13, 24, 25). Furthermore, inactivation of catalase induced the autophagic elimination of altered mitochondria (26). In the yeast \( \text{Saccharomyces cerevisiae} \), the alteration of \( F_{0}F_{1} \) ATPase biogenesis in a conditional mutant has been

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shown to trigger autophagy (27). Alterations of mitochondrial ion homeostasis caused by the inactivation of the K⁺/H⁺ exchanger was shown to cause both autophagy and mitophagy (28). We have reported that treatment of cells with rapamycin induced early ROS production and mitochondrial lipid oxidation that could be inhibited by the hydrophobic antioxidant resveratrol (29). Furthermore, resveratrol treatment impaired autophagic degradation of both cytosolic and mitochondrial proteins and delayed rapamycin-induced cell death, suggesting that mitochondrial oxidation events may play a crucial role in the regulation of autophagy. This existence of regulation of autophagy by ROS has received molecular support in HeLa cells (30): these authors showed that starvation stimulated ROS production, namely H₂O₂, which was essential for autophagy. Furthermore, they identified the cysteine protease hsAtg4 as a direct target for oxidation by H₂O₂. This provided a possible connection between the mitochondrial status and regulation of autophagy.

Investigations of mitochondrial autophagy in nitrogen-starved lactate-grown yeast cells have established the existence of two distinct processes: the first one occurring very early, is selective for mitochondria and is dependent on the presence of the mitochondrial protein Uth1p; the second one occurring later, is not selective for mitochondria, is not dependent on Uth1p, and is not a form of bulk microautophagy (3). The absence of the selective process in the Δuth1 mutant strongly delays and decreases mitochondrial protein degradation (3, 12). The putative protein phosphatase Aup1p has been also shown to be essential in inducing mitophagy (31). Additionally several Atg proteins were shown to be involved in vacuolar sequestration of mitochondrial GFP (3, 12, 32, 33). Recently, the protein Atg11p, which had already been identified as an essential protein for selective autophagy has also been reported as being essential for mitophagy (33).

The question remains as to identify of the signals that trigger selective mitophagy. It is particularly intriguing that selective mitophagy is activated very early after the shift to a nitrogen-deprived medium (3). Furthermore, selective mitophagy is very active on lactate-grown cells (with fully differentiated mitochondria) but is nearly absent in glucose-grown cells (3). In the present paper, we investigated the relationships between the redox status of the cells and selective mitophagy, namely by manipulating glutathione. Our results support the view that redox imbalance is a trigger for the selective elimination of mitochondria.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains, Plasmids, and Growth Conditions—* W303-1B (MATα ade2, his3, leu2, trp1, ura3) was used as the parental strain of the mutant Δuth1 (MATα ade2, his3, leu2, trp1, ura3, uth1::TRP1) (34). For measurement of alkaline phosphatase activity, the PHO8 locus was replaced with PHO8Δ60 by transformation with an HindIII fragment of plasmid pTN9 (a gift from Y. Ohsumi, NIBB Okazaki, Japan) bearing PHO8Δ60 as described previously (35). For fluorescent microscopy experiments, cells were transformed with plasmid pGAL-CLbGFP containing the precursor of the mitochondrial cytochrome c oxidase fused to GFP, under the control of a GAL1/10 promoter (36). The strain Δgsh2 carrying the deletion gsh2::kanMX4 and its parent BY4742 (MATα, his3, leu2, lys2, ura3) were from the Euroscarf collection.

Yeast cells were grown aerobically at 28 °C in a minimal medium containing 0.175% YNB (yeast nitrogen base without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 0.1% potassium phosphate, 0.2% Drop-Mix, 0.01% autotrophic requirements (pH 5.5) supplemented with 2% D-lactate as a carbon source. Nitrogen starvation medium (SD-N) contained 0.175% YNB without amino acids and ammonium sulfate, supplemented with 2% lactate as a carbon source.

*Western Blot Analyses—* Protein sample preparations, SDS-PAGE, and Western blots were previously described (34). Primary antibodies were rabbit polyclonal anti-yeast Atp6p (1/10,000), mouse monoclonal anti-yeast porin (Molecular Probes; 1/5,000), mouse monoclonal anti-yeast phosphoglycerate kinase (Molecular Probes, 1/5,000), and mouse monoclonal anti-GFP (Roche, 1/5,000). Peroxidase-coupled secondary anti-mouse and anti-rabbit antibodies (Jackson Laboratories) were used at 1/5,000. ECL+ (GE Healthcare) was used for detection. Scanning of non-saturated Western blots were quantified with ImageJ software.

*Alkaline Phosphatase Assay—* The alkaline phosphatase activity assay using α-naphyl phosphate as a substrate was performed as described previously (12). Fluorescence intensity was measured at 472 nm (excitation at 345 nm) in a Safas Xenius spectrofluorimeter. Protein concentration was measured with the Lowry method. Activities were expressed as arbitrary fluorescence units per minute per mg of proteins.

*Epifluorescence Microscopy—* To induce mtGFP expression, cells carrying the pGAL-CLbGFP plasmid were grown over-night in the appropriate medium supplemented with 0.5% galactose. GFP was visualized on an epifluorescence microscope (Leica Microsystems DM-LB). The images were acquired with a SIS camera and processed with Corel Draw 9.0 suite software.

*Transmission Electron Microscopy—* For electron microscopy experiments, cells were grown and starved in the presence of 1 mM PMSF to prevent the rapid proteolytic degradation of autophagic bodies. Harvested cells were placed on the surface of Formvar-coated copper grids (400 mesh). Each loop was quickly submersed in liquid propane (−180 °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). Ultrathin sections were stained with lead citrate. Observations were performed on a Philips Tecnai 12 Biotwin (120 kV) electron microscope.

*Glutathione Measurement—* 6 × 10⁷ cells were harvested and the pellet was resuspended in 350 μl of 3.4% metaphosphoric acid. Glass beads (0.4 mm diameter) were added and cells were vortexed (4 × 30 s) and then centrifuged for 2 min at 10,000 × g. Supernatants were used to measure reduced glutathione (GSH) and glutathione disulfide (GSSG) by reverse-phase high pressure liquid chromatography with electrochemical detection (37).
Glutathione-mediated Regulation of Mitophagy

Cell Respiration—Oxygen consumption by growing or nitrogen-starved cells was measured polarographically at 28 °C using a Clark oxygen electrode in a 1-ml thermostatically controlled chamber. Respiratory rates (\( \text{O}_2 \)) were determined from the slope of a plot of \( \text{O}_2 \) concentration versus time.

\( \text{NAD(P)H} \) and \( \text{NAD(P)}^+ \) Measurements (38)—\( \text{NAD(P)H} \) was measured in neutralized methanolic KOH extracts. \( \text{NAD(P)H} \) disappearance was fluorometrically monitored after the addition of dihydroxyacetone phosphate and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) for NADH or \( \alpha \)-cetoglutarate and glutamate dehydrogenase (EC 1.4.1.3) for NADPH. \( \text{NAD(P)}^+ \) was measured in neutralized HClO\(_4\) extracts. \( \text{NAD}^+ \) and \( \text{NADP}^+ \) were fluorometrically monitored as the formation of dihydroethidium, in the presence of various known antioxidants, namely NAC, Tiron, L-ascorbic acid, and resveratrol. Mitophagy was monitored after 24 h of starvation by fluorescent microscopy of mtGFP (Fig. 1).

Under growth conditions, lactate-grown cells exhibited a well differentiated mitochondrial network. Under conditions of physiologically induced autophagy by nitrogen starvation, the appearance of patches of mtGFP at the periphery of the cells, and the simultaneous disappearance of the mitochondrial network were observed. These changes in mitochondrial morphology were followed by extensive delivery of mtGFP into vacuoles: pictures taken after 24 h nitrogen starvation revealed that about 95% of cells had mtGFP in the vacuoles. Tiron, L-ascorbic acid, and resveratrol had no effect on this process. On the contrary, in the absence of NAC, the mitochondrial network was still disrupted but only 4% of cells had mtGFP in the vacuoles. No increase in vacuolar delivery of mtGFP was observed even after 72 h of nitrogen starvation (data not shown).

To examine the possibility that NAC could be a source of nitrogen for starved cells, each of 20 amino acids was individually assayed for a possible effect on vacuolar delivery of mitochondrial proteins by nitrogen starvation. Results showed that cysteine had the same effect as NAC, whereas none of the other amino acids had any effect, as exemplified in Fig. 2 for glutathione, asparagine (which are efficient sources of nitrogen for yeast), and proline (which is poorly utilized as source of nitrogen) (39). This showed that the protective effects of NAC and cysteine on mitophagy are not related to nitrogen supply.

Furthermore, to confirm that NAC actually impaired mitophagy, the hallmarks of this process were investigated. The amounts of two mitochondrial proteins localized in the outer membrane (porin, Por1p) and inner membrane (subunit 6 of ATP synthase, Atp6p) were followed by Western blot analysis, in comparison to a cytosolic protein, phosphoglycerate kinase (Pgk1p) (Fig. 3, A and B). After 24 h of nitrogen starvation, the content of both mitochondrial proteins was depressed, whereas the amount the cytosolic protein was only marginally affected. The degradation of the two mitochondrial proteins was largely impaired when cells were starved in the presence of NAC. In the presence of the hydrophobic antioxidant resveratrol, mitochondrial proteins were still degraded, in accordance with results from fluorescence microscopy (Fig. 1). We also followed the degradation of the cit1-GFP fusion protein by Western blot analysis indicated a significant effect of NAC (\( p < 10^{-5} \)).
during nitrogen starvation. GFP is resistant to proteolysis and the vacuolar delivery of fusion proteins with GFP was shown to be associated to a size reduction corresponding to the degradation of the fused fragment (33). We observed cleavage of fusion protein cit1-GFP to GFP during nitrogen starvation, which was inhibited both by PMSF (inhibitor of vacuolar proteases) and NAC (Fig. 3C). This further confirmed the protective effect of NAC on autophagic mitochondrial proteins degradation.

Electron microscopy of nitrogen-starved lactate-grown cells has previously revealed the existence of two distinct processes for mitochondrial autophagy (3). The first process was characterized by the early and transient appearance of vacuoles/mitochondria contacts and the later appearance of mitochondria-containing vesicles inside the vacuoles. The second process involved a late and non-exclusive engulfment of mitochondria with a significant portion of surrounding cytosol (non-selective microautophagy). After 2 h of nitrogen starvation under lactate as the carbon source, a maximum of 85% of cells displayed vacuole/mitochondria contacts (Figs. 4 and supplemental S1). As previously reported (3), this proportion decreased to 35% after 3 h and completely disappeared after 4 h. These vacuole/mitochondria contacts were early and transient events. After 3 h of starvation, the presence of mitochondria within vacuoles was visualized in 75% of the cells. However, in the presence of NAC, 13% of cells exhibited mitochondria/vacuoles contacts after 2 h of starvation and 13% of cells showed mitochondria sequestration in vacuoles after 3 h of starvation. These observations suggested that in the presence of NAC both processes (mitochondria/vacuoles contacts and mitochondria sequestration in vacuoles) were strongly impaired, although not completely abolished.
FIGURE 4. NAC prevents mitophagy. For electron microscopy experiments, cells were starved in the presence of 1 mM PMSF, as described under “Experimental Procedures.” The hallmarks of mitophagy (contacts between mitochondria/vacuoles and mitochondria sequestered within vacuoles (3)), were investigated in cells starved for 2 or 3 h. The proportion of cells (%) showing each type of event was traced on at least 250 randomly selected cells for each condition (mito, mitochondria; vac, vacuole). Error bars indicate S.D.

Moreover, after 3 h of starvation, the average number of mitochondria sequestered in vacuoles was 1–2 units/slice in the presence of NAC compared with 5–7 units/slice in the absence of NAC (data not shown). These data confirmed that autophagic mitochondrial degradation was inhibited by NAC.

NAC Does Not Inhibit Nonselective Autophagy—We investigated if the non-selective autophagic process was altered by NAC. A well established biochemical method to measure non-selective autophagy is to follow vacuolar delivery and the further activation of Pho8Δ60p, a cytosolic variant of alkaline phosphatase Pho8p. The full-length protein Pho8p is exported to the vacuoles via the normal secretory pathway, where protease B-dependent cleavage of the C-terminal propeptide leads to the activation of the alkaline phosphatase activity. The N-terminal truncated mutant Pho8Δ60p cannot be exported and remains inactive in the cytosol. Upon induction of autophagy, it is sequestered by autophagosomes (macroautophagy) or vacuole invaginations (microautophagy) and delivered to the vacuole where activation takes place. The level of alkaline phosphatase activity in yeast cells where Pho8Δ60p has been substituted for Pho8p is therefore a quantitative measurement of non-selective autophagy.

The alkaline phosphatase activity was measured in lactate-grown Pho8Δ60p-bearing cells submitted to nitrogen starvation, in the absence or presence of NAC (Fig. 5A). Alkaline phosphatase activity increased, independently of the presence of NAC, showing that this molecule did not inhibit non-selective autophagy.

To confirm this observation, electron micrographs were traced for the presence of hallmarks of non-selective autophagy. We have previously reported that macroautophagy is nearly absent from lactate-grown cells submitted to nitrogen starvation and that only hallmarks of microautophagy (direct sequestration of surrounding cytoplasm by the vacuole and appearance of autophagic vesicles) could be observed under these conditions (3). The characteristics of non-selective microautophagy have still been observed in the presence of NAC, further supporting the hypothesis that non-selective autophagy remained active in the presence of the molecule (Fig. 5B).

The Role of Glutathione Metabolism—NAC has been shown to act as an antioxidant through at least two different processes: (i) it scavenges ROS through the reaction with its thiol group (40) and (ii) it stimulates glutathione synthesis after being converted to cysteine. To discriminate between these two effects, different experiments were carried out.

ROS production during nitrogen starvation was measured as the conversion of non-fluorescent dihydroethidium to fluorescent ethidium. Because cellular respiration strongly decreased when cells were submitted to starvation (Table 1, W303), ROS production was normalized to the rate of oxygen consumption (Fig. 6): this ratio markedly increased after 6 and 24 h of starvation. Moreover, NAC did not affect this increase. This result suggested that the effect of NAC on mitophagy did not occur through its ROS scavenging properties. This observation was confirmed by comparing the effects of the D- and L-stereoisomers of N-acetylcysteine. N-Acetyl-D-cysteine has the same scavenging properties as the N-acetyl-L-cysteine (NAC), but cannot enter the glutathione metabolic pathway (41). Contrary to the L-stereoisomer, the D-stereoisomer was without effect on sequestration of the mitochondrial GFP in the vacuoles following nitrogen starvation, because 96% of cells had mtGFP in the vacuoles after 24 h of starvation. This further suggested that the action of N-acetyl-L-cysteine on mitophagy involved its effect on glutathione metabolism but not its scavenging properties.

Glutathione is known to play a key role in the regulation of intracellular signaling, the maintenance of redox status, and the protection against oxidative stress (42). One of the critical factors regulating glutathione metabolism in living cells is the availability of its precursor L-cysteine, because there is only a small pool of this amino acid available to sustain a much larger metabolically active GSH pool (43, 44). The acetylation of the amine function of NAC makes it more permeant than L-cysteine and a better precursor for glutathione metabolism. Consequently, the main effect of NAC might be to fill the L-cysteine pool for glutathione biosynthesis.

NAC prevents mitophagy.
Glutathione-mediated Regulation of Mitophagy

After 2 h of nitrogen starvation, the amount of intracellular GSH decreased by 24% as compared with that measured under growth conditions (Fig. 7, Table 1). This rapid, although modest, decline of the GSH pool was in accordance with observations done by Mehdi and Penninckx (45) on glucose-grown cells submitted to nitrogen starvation. After this initial decrease, the decline of GSH content was moderate because it only reached 40% after 24 h of nitrogen starvation (Fig. 7). As expected, the addition of NAC not only maintained, but strongly increased the intracellular pool of GSH for at least 24 h of starvation (Fig. 7).

To investigate if NAC acted on mitophagy through the increase of GSH, we assayed its effect in the presence of ethanycrynic acid: this drug conjugates to GSH through glutathione S-transferase and consequently reduces the intracellular GSH pool (46). Its effect on the inhibition of nitrogen starvation-induced delivery of mtGFP to the vacuoles by NAC was examined (Fig. 8A). Ethanycrynic acid fully impaired the protective effect of NAC on mitochondrial sequestration. A similar effect was observed when L-cysteine was used instead of NAC (data not shown).

To further manipulate glutathione metabolism, we used mutants of glutathione metabolism. Glutathione biosynthesis is catalyzed in two steps, γ-glutamylcysteine synthase and glutathione synthetase, encoded by the genes GSH1 and GSH2, respectively. In contrast to the gsh1 mutant, which is unable to grow on non-fermentable carbon sources, the gsh2 mutant exhibited growth rates similar to that of wild type on galactose/lactate medium. Because mitophagy could be detected only in cells grown on a non-fermentable carbon source, the gsh1 mutant could not be used and we limited the study to the gsh2 mutant. The inactivation of the GSH2 gene led to the absence of detectable glutathione (47, 48) and the accumulation of γ-glutamylcysteine (γ-Glu-Cys) (49). The Δgsh2 strain displayed a significantly higher rate of nitrogen starvation-induced vacuolar delivery of mtGFP than the wild type (Fig. 8B). In contrast to the wild type, addition of NAC did not arrest mtGFP sequestration in vacuoles in Δgsh2 strain (Fig. 8B). However, NAC still had some inhibitory effect possibly due to the accumulation of γ-Glu-Cys acting like a modulator of redox potentials in cells (49). A protective effect of NAC on wild type (but not on Δgsh2) was observed when it was added before starvation and then removed at the time of starvation, showing that the accumulation of GSH before starvation was enough to partially prevent mitophagy (supplemental Fig. S2).

**TABLE 1**

Effect of NAC on cellular metabolic parameters during nitrogen starvation

|          | JO₂     | NADH/NAD⁺ | NADPH/NADP⁺ | GSH       | GSSG      |
|----------|---------|-----------|-------------|-----------|-----------|
|          | natO/min/10⁷ cells | mmol/10⁷ cells |
| W303     |         |           |             |           |           |
| Growth   | 41.4 ± 4.5  | 0.28 ± 0.05 | 6.2 ± 1.0   | 12.3 ± 1.3 | 0.031 ± 0.030 |
| Nitrogen starvation | 6.1 ± 2.1  | 0.53 ± 0.06 | 38.0 ± 7.0  | 9.4 ± 1.0  | 0.096 ± 0.074 |
| Nitrogen starvation + NAC | 4.3 ± 1.6  | 0.15 ± 0.05 | 8.3 ± 2.0   | 24.0 ± 5.0 | 0.314 ± 0.092 |
| Mu107    |         |           |             |           |           |
| Growth   | 36.5 ± 4.0  | 0.31 ± 0.05 | 1.7 ± 1.0   | 9.4 ± 1.5  | 0.238 ± 0.055 |
| Nitrogen starvation | 6.9 ± 2.2  | 0.51 ± 0.06 | 19.7 ± 7.0  | 8.2 ± 1.0  | 0.282 ± 0.138 |
| Nitrogen starvation + NAC | 4.2 ± 2.1  | 0.35 ± 0.05 | 1.8 ± 2.0   | 12.9 ± 0.5 | 0.370 ± 0.042 |

Glutathione-mediated Regulation of Mitophagy

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To further test the involvement of glutathione in the inhibition of mitophagy, we assayed the effect of a cell-permeable derivative of glutathione, glutathione monoethyl ester, which increases the intracellular GSH concentration both in mammalian and yeast cells (50, 51). As shown in Fig. 8C, glutathione monoethyl ester strongly inhibited mtGFP delivery to the vacuole, although less efficiently than NAC. This confirmed that the effects of NAC and glutathione were similar (although not identical), thus supporting the hypothesis that NAC acted on mitophagy through glutathione metabolism.

We further investigated the effect of NAC on cellular redox balance during starvation, by measuring the reduced/oxidized ratios of NADH/NAD⁺ and NADPH/NADP⁺ couples (Table 1, W303). These ratios increased during nitrogen starvation in accordance with the decrease in respiration rate. This allowed correlating the decline in respiratory capacities of the cells with an increase in the content of reduced coenzymes. The addition of NAC to starved cells restored the ratios of NADH/NAD⁺ and NADPH/NADP⁺ to their levels measured under the growth conditions, despite the still low respiration rate. This
suggested that filling the glutathione pool by NAC relieved NADPH/NADH reoxidation limitations. As a matter of fact, GSSG was nearly undetectable in the absence of NAC, but was measurable in its presence. However, in any case, GSSG remained very low as compared with GSH.

Glutathione-mediated Regulation of Mitophagy Is Uth1p-dependent—We have previously reported that the mitochondrial protein Uth1p is involved in the selective process of mitophagy; indeed, its inactivation prevented the formation of vacuoles/mitochondria contacts and the appearance of intra-vacuolar autophagic vesicles containing mitochondria exclusively. However, a degradation of mitochondria by non-selective microautophagy may still take place (3). To further investigate if glutathione acted on selective mitophagy, we compared the effects of ethacrynic acid on a UTH1-deficient mutant and its parental strain (Fig. 9). In the absence of NAC, the addition of ethacrynic acid significantly stimulated the starvation-induced delivery of mtGFP in the vacuoles of the parental strain (after 8 and 14 h). On the opposite, no effect of ethacrynic acid was observed in the UTH1-deficient mutant. It should be noted that behavior of the UTH1-deficient mutant was not caused by a difference in glutathione content (Table 1, Δuth1). Moreover, the same effects on mitochondrial metabolism (respiration rate and NADH/NADP+ NADPH/NADP+ ratios) were observed in both wild type and Δuth1 strains (Table 1). Consequently, the different behaviors of wild type and Δuth1 strains regarding glutathione modulation could not be explained by a difference in their redox status. More likely, the fact that the Δuth1 strain was not able to do selective mitophagy could explain its insensitivity to ethacrynic acid.

Besides that massive mitophagy induced during nitrogen starvation, mitophagy also emerged when the cells reached the stationary phase (Fig. 10; see also Refs. 31 and 33). The addition of ethacrynic acid in growth medium accelerated vacuolar delivery of mtGFP, as shown after a 48-h growth. Ethacrynic acid only affected mitophagy, as it had no effect of the activity of Pho8460, which depends on non-selective autophagy (data not shown). Furthermore, under conditions of nitrogen starvation, the low level of mtGFP delivery to the vacuoles observed in UTH1-deficient cells in stationary phase was not increased by ethacrynic acid (Fig. 10).

DISCUSSION

Mitochondria have a central role in the control of life, as these organelles are indispensable to generate the energy required for cell function. They consume large amounts of molecular oxygen and the mitochondrial respiratory chain is the major source of intracellular ROS generation (17). Numerous studies have emphasized the role of ROS in the regulation of autophagy (see Ref. 52 for review). Furthermore, a recent study reported direct regulation of one of the components of the molecular machinery of macroautophagy, hsATG4, by H$_2$O$_2$ (30). Another report showed that down-regulation of catalase was a trigger of macroautophagy (26).

However, ROS production might not be the only redox-related event involved in regulation of autophagy. Numerous reports have indicated that maintenance of the glutathione concentration is a priority for cells placed under conditions where the precursors of glutathione synthesis (cysteine, glutamate, and glycine) are hardly available. For example, in liver from fasted rats, the level of glutathione decreased during the first day but was further maintained at about half the initial level (53). In methionine-restricted rats, the level of glutathione was maintained in most tissues, whereas the plasma concentration was strongly depressed (54). In human muscle, a 3-day fasting followed by re-feeding induced changes in amino acid concentration but not in glutathione concentration (55). Similar observations have been done in microorganisms. In starvation-resistant marine bacteria Vibrio sp. submitted to days long
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starvation, the initial drop in intracellular glutathione was followed by maintenance of glutathione for several days. Moreover, the addition of the three glutathione precursors favored the maintenance of a high level of glutathione instead of protein synthesis (56). Similar conclusions could be drawn from experiments in glucose-grown yeast submitted to nitrogen starvation, which maintained about 40% of their glutathione content (45). The decrease of glutathione content has been suggested to be related to up-regulation of CII2, encoding γ-glutamyl transpeptidase, the main glutathione-degrading enzyme (57).

In the present report, we observed that manipulating the intracellular glutathione content modulated mitophagy: (i) nitrogen starvation was associated to an early decrease of GSH content; (ii) the addition of NAC maintained (and actually increased) the intracellular level of GSH and prevented mitophagy; (iii) the depletion of GSH by ethacrynic acid (48) reversed the protective effect of NAC on mitophagy; (iv) the addition of a cell-permeable form of glutathione had a similar effect as NAC.

Glutathione is involved in different major biological functions (see Refs. 58 and 59 for reviews). It participates to the maintenance of the intracellular redox potential, as the glutathione disulfide-glutathione redox couple (GSSG/2GSH) is the major thiol-disulfide redox buffer of the cell (46, 60) and is directly correlated to NAD(P)H/NAD(P)⁺ ratios. We have observed that nitrogen starvation was associated with an imbalance of the redox state of the cell, as measured by an increase of NAD(P)H/NAD(P)⁺ ratios, in accordance with the decrease of respiration rate. This reduced intracellular environment associated with inactive mitochondria and the presence of molecular oxygen would represent a potentially harmful situation for the cells. This would justify the elimination of mitochondria by selective mitophagy. The stimulation of glutathione synthesis (by NAC and cysteine) restored NAD(P)H/NAD(P)⁺ ratios, which would possibly relieve the requirement for mitochondria elimination. As a matter of fact, we have never observed any selective mitophagy on glucose-grown cells (3), where the activity of mitochondria, and the potential danger they might represent, is low.

Like nitrogen starvation, ethacrynic acid induced the decrease of the intracellular glutathione pool that could affect the redox state of the cell and more particularly the redox state of mitochondria. This glutathione decrease could be a trigger for selective mitophagy. The specificity of the effect of glutathione on selective mitophagy was clearly demonstrated by the use of the LITH1-deficient mutant. In this mutant, nitrogen starvation leads to similar effects as in wild type: a modest decrease in the glutathione pool, decrease in mitochondrial respiration, increase of NAD(P)H/NAD(P)⁺ ratios, and restoration of NAD(P)H/NAD(P)⁺ ratios by NAC addition. However, induction of selective mitophagy was impaired in the mutant strain and consequently ethacrynic acid was without effect. This was in accordance with the observation that NAC did prevent the appearance of hallmarks of mitophagy (vacuoles/mitochondria contacts and mitochondria-containing autophagic vesicles) but only had a marginal effect on non-selective autophagy.

On the other hand, glutathione can modulate the activity of a variety of different proteins via S-glutathionylation of cysteine sulphydryl groups and we cannot rule out the hypothesis that thiol-containing proteins targeted by glutathione would play a function in the regulation of mitophagy. Mitophagy might be finely tuned by subtle changes in the concentration and compartmentalization of glutathione and additional investigations are needed to refine this point. Interestingly, it has been reported that, even before the level of glutathione has begun to decrease, aged mice hardly maintain the glutathione level following fasting (61). It is tempting to speculate that this deficiency is correlated, at least partly, to a decline in autophagic activities in aged animals (14–16, 62). Molecular studies in yeast will help to investigate this exciting hypothesis.

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