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Accumulation of dodecyltriphenylphosphonium in mitochondria induces their swelling and ROS-dependent growth inhibition in yeast

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Abstract Hydrophobic cations with delocalized charge are used to deliver drugs to mitochondria. However, micromolar concentrations of such compounds could be toxic due to their excessive accumulation in mitochondria. We studied possible pathophysiological effects of one such cation, i.e. dodecyltriphenylphosphonium (C_{12}-TPP), in the yeast *Saccharomyces cerevisiae*. First, we found that C_{12}-TPP induces high-amplitude mitochondrial swelling. The swelling can be prevented by addition of protonophorous uncoupler FCCP or antioxidant alpha-tocopherol, but not other tested antioxidants (N-acetylcysteine and Trolox). Second, FCCP prevents ROS-sensitive fluorescent dye (dichlorofluorescein diacetate) staining of yeast treated with C_{12}-TPP. We also showed that all tested antioxidants partially restore the growth inhibited by C_{12}-TPP. The latter points that ROS rather than the mitochondria swelling limit the growth rate.

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Abbreviations

C_{12}-TPP	dodecyltriphenylphosphonium
CFU	Colony forming units
EM	electron microscopy
H_2DCF-DA	dichlorofluorescein diacetate
HRP	Horseradish peroxidase
ROS	reactive oxygen species
RTG	retrograde response genes
NAC	N-acetylcysteine

Introduction

Targeting drugs to specific intracellular compartments could significantly improve their efficiency and minimize the side effects. For instance, delivery of the antioxidant plastoquinone to mitochondria, which are considered as the major source of reactive oxygen species, provides a variety of physiological effects on the cellular, tissue, and organismal levels (Skulachev et al. 2009). Dodecyltriphenylphosphonium (C_{12}-TPP) is commonly used for such delivery: due to its delocalized positive charge, the concentration of this compound can be increased by up to 10^4 fold in mitochondria of a live cell (see Severin et al. 2010, Skulachev et al. 2009) compared to the concentration in the medium. Therefore, one can expect that even micromolar concentration of such compound in the media could disturb mitochondrial functioning and induce swelling of the mitochondria matrix. However, the potential

effects of overdose of this compound at the cellular level have not yet been studied in detail.

Mitochondrial swelling is usually associated with pathological processes in eukaryotic cells. Mitochondria normally form branched networks, where processes of fusion and fission of mitochondria are balanced (see review Okamoto and Shaw 2005; Cerveny et al. 2007). In higher eukaryotes swelling is usually associated with opening of the permeability transition pore, which leads to a disturbance in matrix/cytoplasm ion homeostasis (Kaasik et al. 2007). Because the yeast *S. cerevisiae* lacks the mammalian-like permeability transition mechanism (Kovaleva et al. 2010), it can be used as a relatively simple experimental system to study mitochondrial swelling (Nowikovsky et al. 2007; Knorre et al. 2008).

Recently we showed that C_{12}-TPP induces formation of hydrogen peroxide and subsequent collapse of the mitochondrial reticulum in *S. cerevisiae* (Knorre et al. 2010). This change in mitochondrial morphology is not associated with a decrease in cell viability, and in the case of dodecyltriphenylphosphonium (C_{12}-TPP) it can be partially prevented by addition of the antioxidant alpha-tocopherol. On one hand, the latter indicates that ROS are responsible for the change in the mitochondria matrix volume. On the other hand, C_{12}-TPP is a hydrophobic ion that is able to penetrate biological membranes and accumulate in mitochondria due to its positive charge. Thus, it is also possible that the change in mitochondrial volume is a direct result of an excessive accumulation of C_{12}-TPP in the mitochondrial matrix. Indeed, it was recently shown that lipophilic cations like C_{12}-TPP cause swelling of isolated yeast mitochondria (Sukhanova et al. 2010).

Here we report studies of the functional relationships between mitochondrial swelling and ROS production induced by C_{12}-TPP.

Methods

We used W303 (mat alpha) laboratory strain of *Saccharomyces cerevisiae* in this study and *sod1Δ* (*sod1..KanMX4*), *sod2Δ* (*sod2..KanMX4*), and *rho*^{0} (lacking mitochondrial DNA) isogenic derivates. To visualize mitochondria, the W303 cells were transformed with pYX223 plasmid precleaved with Nhe1 restriction enzyme. The plasmid encodes GFP (green fluorescent protein) fused to the mitochondrial targeting sequence (Westermann and Neupert 2000).

Cells were typically grown in liquid culture up to logarithmic phase in YEP-raffinose (containing 2% D-raffinose as the carbon source) medium as reported by Sherman (2002). The growth rate was assessed by colony forming units in suspension culture grown for a period of 2 hours. Initial cell densities were equalized. Under these experimental conditions the control cells increase their CFU indicator by a factor of 2.6±0.2. Therefore, CFU equal to 40% could be considered as complete growth arrest, with lower values of CFU corresponding to cell death.

To visualize intracellular ROS, yeast cells were treated with 50 μM dichlorofluorescein diacetate (H_2DCF-DA, Sigma-Aldrich). H_2O_2 production was measured fluorometrically using HRP (horseradish peroxidase, Sigma) and Amplex red (Invitrogen) (Zhou et al. 1997). Incubation mixture contained 145 mM NaCl, 5 mM KCl, 10 mM MOPS (pH 7.4), 1 mM potassium phosphate, 0.05% glucose, 2 μM Amplex red, and 5 μg/ml peroxidase. The fluorescence was measured with FluoroMax-3 fluorimeter ($\lambda_{excitation}$=530 nm, $\lambda_{emission}$=590 nm.).

Electron microscopy (EM) was done as described by Yang et al. (2006).

Results

First we studied the effect of various concentrations of C_{12}-TPP on viability/growth of yeast wild type strain. We found that C_{12}-TPP at 10 μM concentration did not kill yeast cells, but it almost completely inhibited their growth (Fig. 1a). Recently we have also shown that the same treatment induces collapse of the mitochondrial reticulum, but it does not kill the yeast cells (Knorre et al. 2010).

Addition of antioxidant (alpha-tocopherol, N-acetylcysteine, or TROLOX) restores growth inhibited by C_{12}-TPP (Fig. 1b). This suggests that the inhibition of growth is at least partially determined by ROS formation induced by C_{12}-TPP. However, only alpha-tocopherol, but not the other tested antioxidants, appeared to prevent the collapse of the mitochondrial reticulum (Fig. 1c). As shown by fluorescence (Fig. 1b) and electron (Fig. 2b and Table 1) microscopy, C_{12}-TPP induces dramatic swelling of the mitochondrial matrix, and alpha-tocopherol addition, while alleviating this effect, still does not completely restore the mitochondrial morphology to the normal state (Fig. 2 and Table 1). But most importantly, other antioxidants, which are more hydrophilic than alpha-tocopherol, rescued the growth of C_{12}-TPP-treated cells without any visible effect on mitochondrial morphology. Thus, such a massive mitochondrial volume increase does not seem to be harmful for the cells, but ROS production appears to be toxic.

Next we tested whether the effects of C_{12}-TPP depend on its mitochondrial localization. It was previously shown that uncouplers dissipate mitochondrial membrane potential and in this way prevent the accumulation of hydrophobic cations in the matrix (Severin et al. 2010). Thus we used FCCP as a tool to inhibit the accumulation of C_{12}-TPP. The concentration of the uncoupler was chosen to be optimal for stimulation of oxygen consumption of intact yeast cells

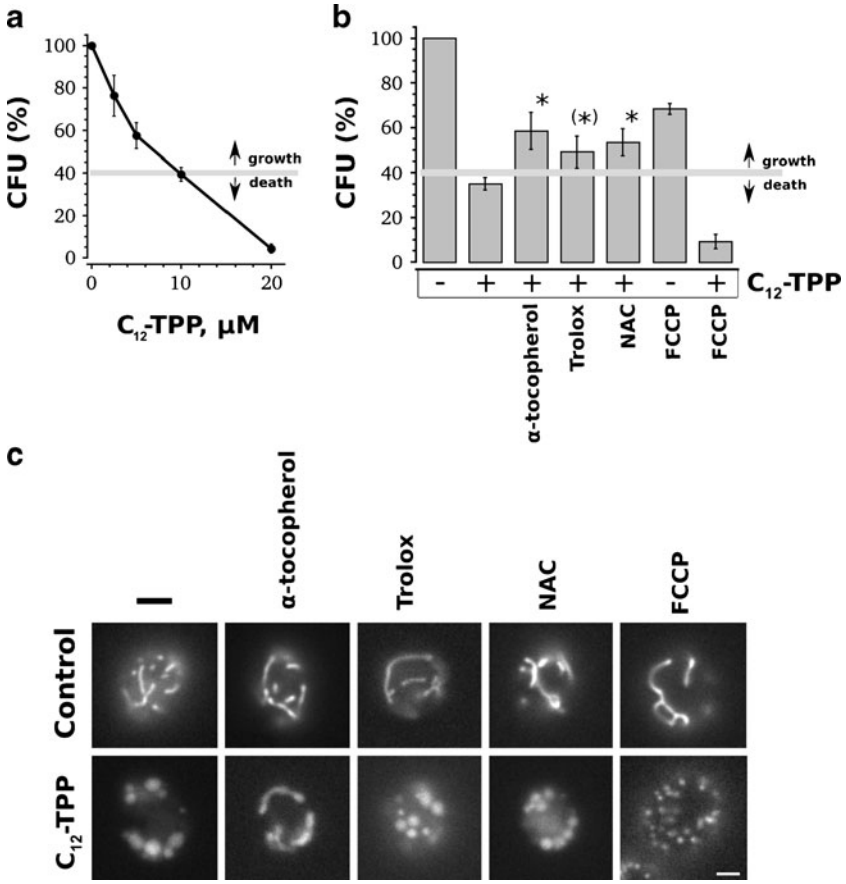

Fig. 1 C_{12}-TPP decreases yeast growth rate that can be partially restored by antioxidants. **a** Effect of various concentration of C_{12}-TPP on colony forming units (CFU) in yeast suspension. **b** Antioxidants alpha-tocopherol, N-acetylcysteine, and/or Trolox protect yeast cells from C_{12}-TPP-induced inhibition of growth. **c** The effect of antioxidants and uncoupler FCCP on C_{12}-TPP-induced mitochondria morphology changes (representative microphotographs). Mitochondrial morphology was visualized by expression of mitochondria-targeted GFP in the control cells. Bar—2 μm. C_{12}-TPP (indicated as "+") was added to 10 μM, alpha-tocopherol to 50 μM, Trolox to 100 μM, N-acetylcysteine (NAC) to 5 mM, and FCCP to 1 μM. **a** Growth rates were assessed by the numbers of colony forming units (see Materials and Methods). Horizontal line corresponds to the number of CFU's at zero time point. (*) $p<0.05$ compared to the control with C_{12}-TPP in the *t*-test ($p<0.06$ for Trolox)

(Pozniakovsky et al. 2005). As expected, FCCP addition inhibited C_{12}-TPP-induced changes in the mitochondrial morphology. Indeed, both fluorescence (Fig. 1c) and electron (Fig. 2f and Table 1) microscopy show the strongly decreased number of swollen mitochondria in the cells treated with the mixtures of C_{12}-TPP and FCCP. Consistent with the idea that the swelling causes ROS production, FCCP treatment inhibited the fluorescence of C_{12}-TPP-treated cells stained with the ROS-activatable dye H_2DCF-DA (Fig. 3a). Unexpectedly, the mixture of C_{12}-TPP and FCCP appeared to be toxic for the cells (Fig. 1b). Possibly, C_{12}-TPP and FCCP, similar to C_{12}-TPP and free fatty acid (Severin et al. 2010) act as an efficient uncoupling pair, decreasing the mitochondria transmembrane potential below the minimum required for cell viability. This idea is supported by the observation of a cumulative effect of C_{12}-TPP and FCCP inducing a proton leak across bilayer planar phospholipid membrane (I. Severina, manuscript in preparation).

What is the source of ROS induced by C_{12}-TPP? Since FCCP prevented DCF staining of C_{12}-TPP-treated cells (Fig. 3a), the accumulation of the cation in the matrix seems to be necessary for ROS production, which suggests the mitochondrial nature of the ROS. We reasoned that C_{12}-TPP accumulated in mitochondria could disturb the respiratory chain and thus elevate superoxide production in the mitochondria matrix. As in Knorre et al. (2010), we used the Amplex Red/Peroxidase system to measure the rate of exogenous hydrogen peroxide formation in the presence of C_{12}-TPP (Fig. 3b). We used higher concentration of C_{12}-TPP (20 μM) to induce H_2O_2 generation to increase the sensitivity of the assay. It was found that C_{12}-TPP-induced ROS production also occurs in *rho*^{0} cells, i.e. in the absence of a functional respiratory chain (Fig. 3c). At the same time, the increase in C_{12}-TPP-induced generation of hydrogen peroxide in *sod2*Δ strain (Fig. 3c) was lower than in the parental strain. In contrast to *sod2*Δ, cells with inactivated

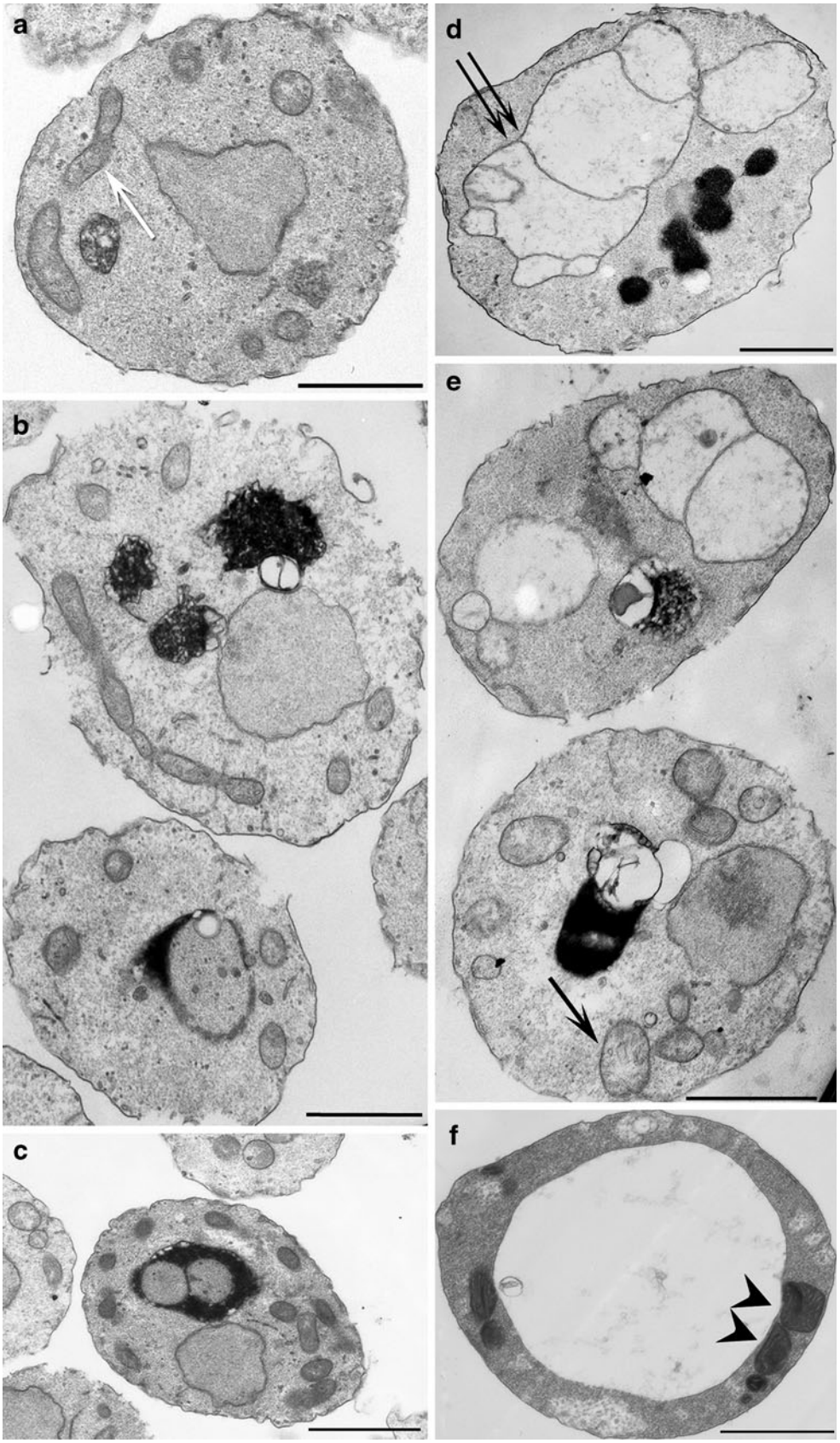

Fig. 2 Effects alpha-tocopherol and FCCP on the ultrastructure of C_{12}-TPP-treated cells. The additions were: **a** no additions; **b** 50 μM alpha-tocopherol; **c** 2 μM FCCP; **d** 10 μM C_{12}-TPP; **e** 10 μM C_{12}-TPP and 50 μM alpha-tocopherol; **f** 10 μM C_{12}-TPP and 2 μM FCCP. The white arrow indicates native-looking mitochondria; black arrow, moderately swollen mitochondria; double black arrow, severely swollen mitochondria; and double black triangles, electron dense mitochondria located in the cell periphery due to the extremely large vacuoles in the cytoplasm. See Table 1 for quantitative analysis of the data. Bar—1 μm

Table 1 Quantitative characterization of yeast mitochondrial swelling based on electron microphotographs. Representative mitochondria are shown in Fig. 2 and indicated by arrows. "Moderate swelling" means that mitochondria are of increased area and lighter/clarified matrix. "High-amplitude swelling" means that mitochondria occupy the area more than tenfold greater than the area of the control ones. The indicated values correspond to the percentage of cells containing at least one swollen mitochondria of either type

Treatment\Level of mitochondrial swelling	Moderate swelling,%	High-amplitude swelling,%	Number of cells analyzed
Control, solvent	0	0	441
C12-TPP, 10 μM	8	79	429
C12-TPP, 10 μM + FCCP, 1 μM	22	22	185
C12-TPP, 10 μM +α-tocopherol, 50 μM	30	56	419
α-tocopherol, 50 μM	0	0	179
FCCP, 1 μM	1	0	50

cytoplasmic superoxide dismutase (*sod1*Δ) were still able to induce hydrogen peroxide formation in response to C_{12}-TPP (Fig. 3c). This result indicates that C_{12}-TPP treatment induces formation of superoxide in the mitochondrial matrix. Indeed, cells lacking matrix superoxide-dismutase are not able to convert matrix superoxide to hydrogen peroxide able to diffuse from the cell and thus being detectable by Amplex Red/HRP. To summarize, these data suggest that C_{12}-TPP induces formation of superoxide on the matrix side of inner mitochondrial membrane.

Discussion

There is much controversy about the relationships between mitochondrial ultrastructure and ROS production. A malfunctioning mitochondrial respiratory chain was claimed to be the major source of ROS, the amounts of which can be sufficient to cause apoptotic or necrotic cell death (reviewed in Lee and Wei 2000; Ott et al. 2007). Conversely, high levels of ROS are associated with abnormal mitochondrial morphology. It is thought that

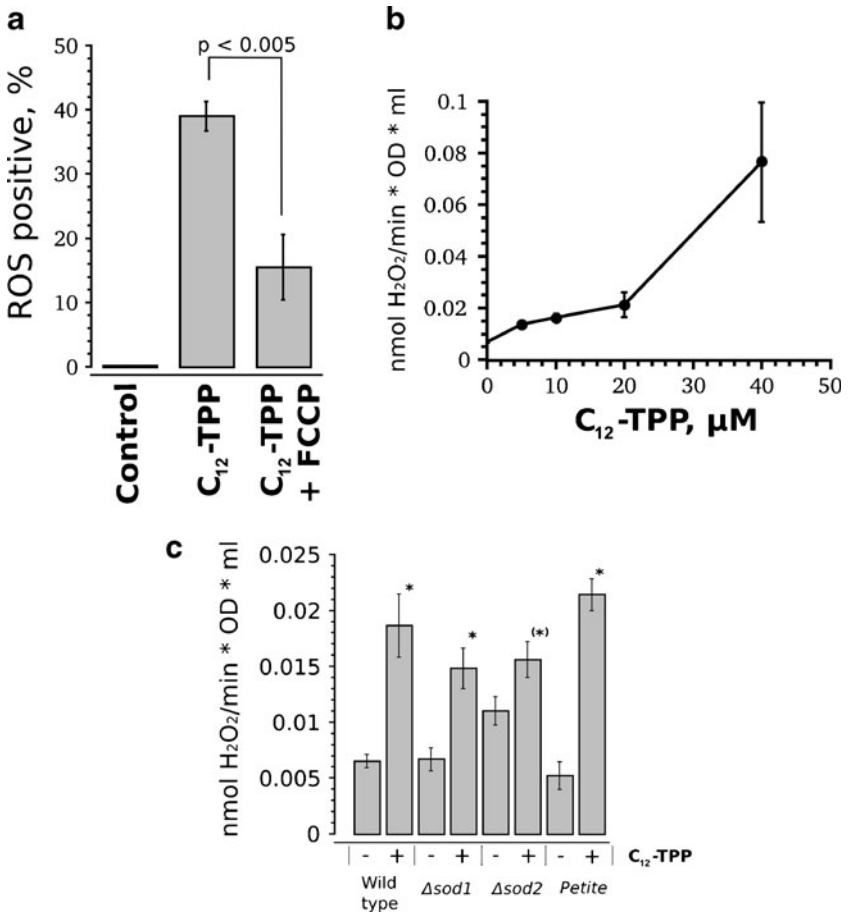

Fig. 3 C_{12}-TPP induces ROS formation. **a** Uncoupler FCCP prevents C_{12} -TPP (10 μM)-induced DCF staining of wild type yeast cells. **b** Effect of various concentrations of C_{12} -TPP on hydrogen peroxide formation. **c** 20 μM C_{12}-TPP (indicated as "+") induces formation of hydrogen peroxide in the wild type yeast cells and also in yeast cells lacking mitochondrial DNA (petite) or superoxide-dismutases (*sod1* or *sod2*). (*) $p<0.001$ compared to the control without C_{12}-TPP according to the *t*-test ($p<0.06$ for *sod2*)

ROS induce permeability transition pore opening, which results in mitochondria swelling (Peng and Jou 2004). At the same time, increased ROS levels promote fragmentation of mitochondria (see Zorov et al. 2005). There are also indications that ROS can promote mitochondrial fusion: α-tocopherol prevents the formation of large megamitochondria in hepatocytes of rats fed with the oxidative stress-inducing agent hydrazine (Antosiewicz et al. 1994).

Our findings allow drawing a model of mitochondria morphology–ROS relationships for the particular experimental system lacking mitochondria permeability transition (Kovaleva et al. 2010). Our data on FCCP/C_{12}-TPP treatments show that mitochondrial abnormalities caused by C_{12}-TPP accumulation in the matrix trigger ROS accumulation. The result showing that N-acetylcysteine or Trolox addition prevents the growth inhibition but not the collapse of the mitochondrial reticulum supports this conclusion.

The most surprising result is that 50 μM alpha-tocopherol not only restores the growth rate but also prevents mitochondria swelling. There are at least two possible reasons for this observation. (1) We used alpha-tocopherol at relatively high concentration [as in Pozniakovsky et al. (2005)]. It is possible that concentrated alpha-tocopherol disturbs the mitochondrial inner membrane and in this way decreases mitochondrial transmembrane potential. As a result, accumulation of C_{12}-TPP is prevented. However, the effect of FCCP in the presence of C_{12} -TPP is principally different: FCCP completely abolishes the swelling and, unlike alpha-tocopherol, FCCP combined with C_{12} -TPP kills the cells. This indicates that uncoupling it is not the major mechanism of action of alpha-tocopherol in our experimental system. (2) It seems more likely that there is a reaction with positive feedback: more ROS—more matrix swelling, and alpha-tocopherol is the most efficient in preventing the ROS effect on mitochondrial membrane due to its extremely high hydrophobicity. The latter conclusion comes from the fact that alpha-tocopherol partially inhibits the swelling.

The most surprising seem to be the indications that the swelling-induced ROS are more harmful for cells than the mitochondrial swelling per se. Indeed, the cells with swollen mitochondria grow as fast as the control cells provided that the ROS are scavenged by antioxidants. This means that swollen mitochondria that accumulated vast amounts of C_{12}-TPP are generally functional. This is supported by our previous observations that C_{12}-TPP at 10 μM concentration does not inhibit respiration (Severin et al. 2010). However, ROS production does not depend on the mitochondrial respiratory chain: it also occurs in *rho*^{0} strain. Moreover, alpha-tocopherol is equally effective in restoration of C_{12}-TPP-induced growth arrest of *rho*^{0} (petite) and *rho*$^+$ cells (data not shown).

There is a well established way for mitochondria to communicate their malfunctioning to the rest of the cell, i.e. the retrograde pathway. This pathway relies on three proteins (Rtg1, 2, and 3), is activated by mitochondria, and mediates the transcriptional response of the cell to mitochondrial stresses (reviewed in Liu and Butow 2006). In our model the involvement of this pathway can be ruled out: null mutation of each RTG does not inhibit C_{12}-TPP-induced ROS generation (data not shown). We suggest that in the case of the major swelling the excessive ROS production causes damage to mitochondria and in this way further stimulates the swelling.

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