Relation between cell death progression, reactive oxygen species production and mitochondrial membrane potential in fermenting Saccharomyces cerevisiae cells under heat-shock conditions

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One sentence summary: Generation of reactive oxygen species under moderate heat shock in glucose-grown Saccharomyces cerevisiae cells is driven by the mitochondrial membrane potential.

ABSTRACT

Moderate heat shock increased reactive oxygen species (ROS) production that led to cell death in glucose-grown Saccharomyces cerevisiae cells. Conditions that disturb mitochondrial functions such as treatment by uncouplers and petite mutation were shown to inhibit ROS production and protects cell from thermal death. Hence, mitochondria are responsible for ROS production and play an active role in cell death. An increase in ROS production was accompanied by hyperpolarization of inner mitochondrial membrane. All agents suppressing hyperpolarization also suppressed heat-induced ROS production. It was supposed that generation of ROS under moderate heat shock in glucose-grown S. cerevisiae cells is driven by the mitochondrial membrane potential.

Keywords: hyperpolarization of inner mitochondrial membrane; ROS; mitochondria; heat shock; cell death

INTRODUCTION

Mitochondria are responsible for reactive oxygen species (ROS) generation in the eukaryotic cell (Kowaltowski et al. 2009; Rigoulet, Yoboue and Devin 2011). The same seems to be true for Saccharomyces cerevisiae cells treated by heat shock. Heat shock induced an increase in ROS production (Davidson et al. 1996; Cao et al. 2013; Fedoseeva et al. 2014), and there is some ground to believe that mitochondria are involved in the ROS production (Davidson et al. 1996; Davidson and Schiestl 2001). However, how mitochondria produce ROS in yeast cells is not well understood. It was supposed that external mitochondrial NADH dehydrogenases Nde1p and Nde2p participate in ROS production under heat-shock conditions in yeast cells. The deletion of NDE1 and NDE2 reduced heat-induced ROS production and protected cells from death (Davidson and Schiestl 2001). The involvement of external NADH dehydrogenase in ROS production in yeast cells has been supported by other researches (Fang and Beattie 2003; Li et al. 2006; Gomes et al. 2013). Nde1p and Nde2p oxidize cytosolic NADH produced during glycolysis, therefore the ability...
of these dehydrogenases to produce ROS is strictly dependent on the availability of cytosolic NADH. The cytosolic NADH/NAD⁺ ratio is neutral in glucose-grown S. cerevisiae cells and increases under respiratory growth conditions, i.e. during growth on non-fermentable carbon sources or in stationary growth phase, when glucose is exhausted and cells have to switch from fermentative to respiratory metabolism (Bakker et al. 2001; Murray, Haynes and Tomita 2011). Apparently for this reason the ability of NDE1 and NDE2 deletions to suppress heat-induced ROS production in yeast cells was demonstrated only for respiring yeast cells (Davidson and Schiestl 2001). Meanwhile, heat shock was shown to induce a more profound increase of ROS production in fermenting S. cerevisiae cells by comparison with respiring cells (Fedoseeva et al. 2014).

The mitochondrial membrane potential (MMP) is generated by proton pumping by the respiratory complexes from matrix of mitochondria to the outside of the inner mitochondrial membrane. Experiments in isolated mitochondria have shown that an increase in MMP was accompanied by a rise in ROS production (Korshunov, Skulachev and Starkov 1997). A similar situation was observed in mammalian cells, and decrease in the mitochondrial potential usually inhibited the ROS production (Suski et al. 2012). It was suggested by V.P. Skulachev that ROS are formed in mitochondria, when the mitochondrial inner membrane potential is high and some components of respiratory electron transport, capable of reducing O₂ to O₂⁻⁻ become long lived (Skulachev 1996). Heat shock was shown to induce a hyperpolarization of inner mitochondrial membrane in yeast (Rikhvanov et al. 2005; Fedoseeva et al. 2012), plant (Rikhvanov et al. 2007; Pyatritsak et al. 2014) and mammalian (Balogh et al. 2005) cells. But whether or not such an association with ROS production remains unknown. Therefore, in the current work, we have explored a link between MMP, ROS production and cell death in S. cerevisiae cells under heat-shock conditions. To do that glucose-grown S. cerevisiae cells have been used. Under these conditions, despite the repression of oxidative phosphorylation by glucose, yeast mitochondria continue to maintain MMP, which is critical for viability (Traba, Satrústegui and del Arco 2009).

MATERIALS AND METHODS

Strains and growth conditions

The yeast S. cerevisiae, parent type strain W303–1B (MATa ade2–1 his3–11, 15 trp1–1 leu2–3112 ural3–1 [rho⁻] ) and its isogenic petite mutant were used. Petite mutant was obtained by ethidium bromide treatment. The mutant phenotype was confirmed (i) by absence of growth in the presence of ethanol, (ii) by absence of oxygen uptake and (iii) by absence of mtDNA after staining with DAPI (4',6-diamidino-2-phenylindole) (Fig. S1, Supporting Information). The cells were maintained at 30 °C on YEPD medium containing 0.5% yeast extract, 1% peptone, 2% glucose and 1.5% agar. The cells were grown at 30°C in 100 ml Erlenmeyer flasks with 25 ml of liquid YEPD or YEP medium (in the latter medium, glucose was substituted by 20 ml 1⁻¹ ethanol). Cells in logarithmic or stationary growth phase were used in experiments.

Count of colony-forming units (CFU)

The effect of ascorbic acid (AsA), 2, 4-dinitrophenol (DNP) and carbonylcyanide m-chlorophenylhydrazone (CCCP) on thermo-tolerance was studied by the addition of agents to yeast cells at 30 or 45 °C. To count CFU, the yeast cells were diluted and plated in a standard way in YEPD medium containing 1.5% agar. After 24–48 h incubation at 30 °C, the CFU were counted, and the data are represented as percentage of control.

Fluorescence microscopy

ROS generation was studied with the use of 50 μM 2’, 7’-dichlorofluorescein diacetate (H₂DCF-DA). The MMP was qualitatively visualized using the potential-dependent cationic dye MitoTracker Orange (MO) at a final concentration of 50 nM. The data were recorded following 30 min incubation of the cells with two dyes. DCF and MO fluorescence were analyzed with the use of an inverted fluorescent microscope AxioVision Z1 (Germany), a digital monochromatic camera AxioCamMRm3 and the software package AxioVision Rel.4.6 designated for image capture and analysis. In total, ten images of each variant were analyzed.

Statistical analysis

All experiments were repeated no less than three times. The data were statistically processed for mean values and standard deviations.

RESULTS

Cell death induced by moderate heat shock depends on ROS production

Heat shock was known to enhance ROS production in yeast cells (Davidson et al. 1996; Cao et al. 2013; Fedoseeva et al. 2014). In accordance with the published data, the treatment of log-phase S. cerevisiae cells grown on glucose at 45 °C for 30 min induced a sharp increase in ROS production determined by elevation of DCF fluorescence (Figs 1a and 2a, Supporting Information). At the same time, heat shock at 45 °C strongly decreased cell viability (Fig. 1b). Increased ROS production was the main cause of cell death under these conditions, as evidenced by the fact that addition of AsA inhibited ROS production (Figs 1a and 2a, Supporting Information) and significantly protected yeast cells (Fig. 1b). Yeast cells growing on glucose obtain energy primarily by fermentation (Bakker et al. 2001). Hence, even under conditions of repression of the main mitochondrial function, i.e. ATP synthesis, the yeast cells produce ROS in response to heat shock 45 °C, which play an important role in heat-induced cell death.

Interestingly that despite the significant protective effect of AsA during treatment at 45 °C (Fig. 1b), in the case of more severe heat shock (50 °C), the ability of AsA to increase the survival was significantly diminished (Fig. S3, Supporting Information). Obtained results confirm our previous suggestion (Rikhvanov et al. 2014) that death of fermenting cells after treatment at 50 °C is largely independent on ROS production. Therefore, to differentiate between two modes of cell death, we have denoted the heat shock at 45 °C, as ‘moderate heat shock’ and heat shock at 50 °C, as ‘severe heat shock’.

Heat-induced ROS production depends on functional yeast mitochondria

Uncouplers, DNP and CCCP, inhibit mitochondrial functions by allowing free conductance of protons across inner mitochondrial membrane thereby dissipating mitochondrial membrane potential. Uncouplers were shown to inhibit ROS production in isolated mammalian mitochondria (Korshunov, Skulachev and Satrústegui 2001). To do that glucose-grown S. cerevisiae cells were diluted and plated in a standard way in YEPD medium containing 1.5% agar. After 24–48 h incubation at 30 °C, the CFU were counted, and the data are represented as percentage of control.
Figure 1. The effect of AsA on ROS generation (a) MMP (c) and cell death (b) induced by moderate (45 °C, 30 min) heat shock. Yeast cells of strain W303–1B were grown in YEPD medium and incubated at 30 or 45 °C in the absence (C, control conditions) or in the presence of 10 mM AsA. DCF and MO fluorescence were measured immediately after treatment. Survival was evaluated by CFU counting after 48 h of incubation at 30 °C.
Figure 2. The effect of DNP on ROS generation (a), MMP (c) and cell death (b) induced by moderate (45°C) heat shock. Yeast cells of strain W303–1B were grown in YEPD medium and incubated at 30 or 45°C in the absence (C, control conditions) or in the presence of DNP (100–100 μM). DCF and MO fluorescence were measured immediately after treatment. Survival was evaluated by CFU counting after 48 h of incubation at 30°C.

Figure 3. The effect of CCCP on ROS generation (a), MMP (c) and cell death (b) induced by moderate (45°C) heat shock. Yeast cells of strain W303–1B were grown in YEPD medium and incubated at 30 or 45°C in the absence (C, control conditions) or in the presence of CCCP (0.5–2 μM). DCF and MO fluorescence were measured immediately after treatment. Survival was evaluated by CFU counting after 48 h of incubation at 30°C.
Figure 4. The effect of petite mutation on ROS generation (a) MMP (c) and cell death (b) induced by moderate (45°C) heat shock. Yeast cells of the parent type W303–1B (PT) and petite mutant were grown in YEPD medium and incubated at 30 or 45°C. Survival was evaluated by CFU counting after 48 h of incubation at 30°C.

ROS production induced by moderate heat shock is correlated with hyperpolarization of inner mitochondrial membrane

Previously, it was shown that mild heat shock which did not produce deleterious effect on survival led to an increase in MMP in yeast cells (Rikhvanov et al. 2005; Fedoseeva et al. 2012). Because in some experimental models the correlation between MMP and mitochondrial ROS production was observed (Korshunov, Skulachev and Starkov 1997; Pozniakovskiy et al. 2005; Suski et al. 2012), it was interesting to know whether or not moderate heat shock, which significantly decreases yeast survival (Fig. 1b), also induces hyperpolarization of the inner mitochondrial membrane. As shown on Figs 2, 3 and 4, heat treatment at 45°C resulted in a significant increase in MMP, as evidenced by an increase in fluorescence MitoTracker Orange, MMP sensing probe. The addition of DNP (Fig. 2c), CCCP (Figs 3c and 4b, Supporting Information) as well as petite mutation (Fig. 4c) effectively suppressed an increase in MMP. It is interesting that AsA also suppressed heat-induced ROS production (Figs 1a and S2a, Supporting Information) and hyperpolarization (Figs 1c and S2b, Supporting Information). Obviously, all conditions which are able to inhibit the heat-induced MMP increase in yeast cells also inhibit ROS production. These results suggest that heat-induced ROS production and progression of cell death are accompanied by hyperpolarization of inner mitochondrial membrane.

The ability of uncouplers and petite mutation to suppress heat-induced ROS production strongly suggests that ROS production in log-phase glucose-grown yeast cells depends on mitochondrial functions. To provide additional evidence, we made an attempt to evaluate the intracellular localization of ROS in yeast cells under heat-shock conditions. To do that, yeast cells were double-stained with H$_2$DCF-DA and MitoTracker Orange and subjected to moderate heat shock. MitoTracker Orange was used for localization of mitochondria in the cells. H$_2$DCF-DA is the most widely used fluorogenic probe for hydrogen peroxide determination (Suski et al. 2012). As a rule, DCF-stained regions in heat-shock-treated samples were found in cytosol. Hydrogen peroxide is a relatively stable metabolite and could diffuse from the site of production. But in some yeast cells DCF fluorescence were colocalized with MitoTracker Orange signal (Fig. 5), confirming that mitochondria are responsible for heat-induced ROS production in S. cerevisiae.

DISCUSSION

An increase in ROS production was shown to be the cause of yeast cell death induced by heat shock (Davidson et al. 1996; Davidson and Schiestl 2001; Cao et al. 2013). Results obtained in the course of the current study clearly support this point of view. Moderate heat shock at 45°C resulted in a rise in ROS production (Figs 1a and S2a, Supporting Information) and cell death (Fig. 1b) in glucose-grown S. cerevisiae cells. The antioxidant AsA
On the contrary, results obtained in the present study give evidence that under condition of repression of the main mitochondrial function, i.e. ATP synthesis, the yeast mitochondria preserve functionality and produce ROS in response to heat shock. It seems that connection between mitochondria and ROS is intricate, as abrogation of mitochondrial function can either induce or suppress ROS production. Moreover, the obtained results clearly suggest a close positive link between ROS production and an increase of MMP. Moderate heat-shock-induced an increase in MMP (Figs 1c and 4c) and such an event was accompanied by a rise in ROS production (Figs 1a and 4a). A similar correlation between MMP and ROS production in S. cerevisiae has been repeatedly reported in literature. In particular, treatment by acetic acid (Ludovico et al. 2002; Amigoni, Martegani and Colombo 2013), α-factor (Severin and Hyman 2002), amiodarone (Pozniakovsky et al. 2005), heterologous expression of human Bax (Gross et al. 2000), mutation Ras2 (Hlavátá et al. 2003), deletion of HXX2 (Amigoni, Martegani and Colombo 2013) and mutations which reduce TOR signaling (Pan et al. 2011) resulted in MMP increase and enhanced ROS production. It is evident that a similar situation occurred in fermenting yeast cells subjected to moderate heat shock. The fact that depolarization of mitochondrial membrane by DNP (Fig. 2c), CCCP (Fig. 3c) or petite mutation (Fig. 4c) led to a dramatic decrease in ROS production strongly suggests that hyperpolarization induced by heat shock is responsible for mitochondrial ROS generation. It should be determined whether or not a close dependence between MMP increase, enhanced ROS production and thermotolerance is valid for respiring yeast cells under heat-shock condition. But inability of low concentrations of DNP and CCCP, which effectively protected fermenting cells (Figs 2 and 3), to increase the thermotolerance of respiring cells (Fig. S5, Supporting Information) suggests against it.

A connection between ROS production and hyperpolarization was earlier shown for S. cerevisiae cells treated by the amiodarone (Pozniakovsky et al. 2005). Authors proposed a scheme of mitochondrial death cascade in yeast (Pozniakovsky et al. 2005). According to the scheme, treatment of cells with amiodarone leads to a rise in cytosolic Ca$^{2+}$ level. Ca$^{2+}$ stimulates the activity of certain respiratory enzymes and increases coupling of respiration and energy conservation. As a result, an elevation of MMP led to an increase of ROS production, which then promotes mitochondrial fragmentation, loss of MMP and progression of cell death (Pozniakovsky et al. 2005). It should be verified whether or not such scenario occurs in glucose-grown yeast cells under condition of moderate heat shock.

It is accepted that mitochondrial ROS production is increased with overreduction of the respiratory chain (Kowaltowski et al. 2009; Rigoulet, Yoboue and Devin 2011). Overreduction could be observed in two cases. First, when the activity of respiratory components is inhibited. For instance, the inhibition of complex III by antimycin A leads to overreduction of ubiquinone (Kowaltowski et al. 2009; Rigoulet, Yoboue and Devin 2011). Second, when the rate of electron input exceeds the ability of respiratory chain to transport them or transport of electrons is slowed. In first case, ROS production would be accompanied by MMP decrease, but in second case ROS production would be associated with MMP increase. Probably, the second mechanism is operating in glucose-grown yeast cells under heat-shock conditions.

**SUPPLEMENTARY DATA**

Supplementary data is available at FEMSEC online.


