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Viability of Saccharomyces cerevisiae cells following exposure to H2O2 and protective effect of minocycline depend on the presence of VDAC

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Abstract

Proteins involved in apoptosis are still a matter of debate. Therefore, we decided to check the effect of the presence of VDAC (voltage dependent anion selective channel) on viability of Saccharomyces cerevisiae cells following their exposure to H2O2 that is known to induce apoptosis both in S. cerevisiae and in mammalian cells. Mitochondria of S. cerevisiae contain only one channel-forming VDAC isoform (VDAC1), which simplifies studies on the channel. Using S. cerevisiae mutant depleted of VDAC1 (termed here VDAC) and the isogenic wild type, we have shown that VDAC is important for protection of S. cerevisiae cells against H2O2 treatment, particularly in exponential growth phase that is known to be more affected by H2O2. The increased viability of H2O2 pretreated exponentially growing cells containing VDAC was accompanied by clear changes of the cytosol redox state and was potentiated by minocycline, an antibiotic of the tetracycline family that displays cytoprotective potency. The protective effect of minocycline also coincided with distinct changes of cytosol redox state. Thus, we conclude that the ability to change the cytosol redox state following exposure to H2O2 or/and minocycline appears to be an intrinsic feature of exponentially growing cells (young cells) containing VDAC. Moreover, the ability seems to be crucial for both cell viability and protective effect of minocycline.

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1. Introduction

Life and death of unicellular organisms are in some cases comparable to those of multicellular organisms. Therefore, the yeast Saccharomyces cerevisiae is often used for studying a variety of complex pathways including cell division, signal transduction and apoptosis (Magherini et al., 2007; Perrone et al., 2008). It is well known that these processes may be influenced by intracellular reduction/oxidation (redox) states since the redox states affect expression, stabilization, localization, accessibility, interactions and activity of involved proteins (Galganska et al., 2010; Liu et al., 2005; Pesaresi et al., 2007; Woodson and Chory, 2008). The mechanism of apoptosis is complex and still remains elusive but available data indicate that pro-apoptotic and anti-apoptotic stimuli may trigger a state of high or low conductance of voltage dependent anion selective channel (VDAC) (Kroemer et al., 2007; Mannella and Kinnally, 2008).

Physiologically, VDAC functions as the major channel allowing passage of metabolites between the intermembrane space of mitochondria and the cytoplasm (Benz, 1994; Blachly-Dyson and Forte, 2001; Colombini, 2004; De Pinto et al., 2003; Lemasters and Holmuhamedov, 2006; Mannella and Kinnally, 2008; Shoshan-Barmatz et al., 2008). The channel may be present as isoforms encoded by separated genes, displaying different channel-forming activities and probably playing different roles. For example, S. cerevisiae mitochondria express two VDAC isoforms, of which only one, encoded by the POR1 gene (VDAC1 or POR1), has been proved to form a channel (Blachly-Dyson et al., 1997; Lee et al., 1998). It has been shown that VDAC plays a crucial role in ATP rationing, Ca2+ homeostasis and execution of mitochondria-mediated apoptosis. Interestingly, we have demonstrated that VDAC1 mediates the cytosol reduction/oxidation (redox) state and influences the activity of antioxidant enzymes (Budzinska et al., 2007; Galganska et al., 2008). Therefore, VDAC may be also important for cell survival following the cell exposure to oxidizing agents such as hydrogen peroxide (H2O2).

On the other hand, if VDAC is involved in antioxidant defense it could also serve as a target of cytoprotectants. Accordingly, we have shown that minocycline, an antibiotic of the tetracycline family that displays cytoprotective potency (e.g., Gieseler et al., 2009), affects activity of...
VDAC as determined in the reconstituted system (Garcia-Martinez et al., 2010).

Therefore, we pretreated wild type and Δpor1 S. cerevisiae cells with H2O2 in the absence or in the presence of minocycline. We observed that VDAC1 (termed here VDAC) was important for viability of S. cerevisiae cells after their exposure to H2O2 as well as for the protective effect of minocycline. The effects of H2O2 and minocycline were distinctly pronounced for exponentially growing cells. Moreover, the effects also coincided with changes of the cytosol redox state and of the content of mitochondrial proteins, namely VDAC and Mge1p (mitochondrial GrpE protein, Laloraya et al., 1994) used as a loading control. Thus, we conclude that exponentially growing cells (young cells) containing VDAC are able to change the cytosol redox state when pretreated with H2O2 or/and with minocycline and the ability appears to be important for both cell viability and protective effect of minocycline.

2. Materials and methods

2.1. Yeast strains, culture conditions and cell viability assay

The following S. cerevisiae strains were used: the isogenic wild type M3 (MATa by2 his4 trp1ade2 leu2 ura3) and VDAC1 (porin1)–depleted mutant M22-2 (Δpor1) containing a deletion of most of the POR1 gene (Blachly-Dyson et al., 1997: Lee et al., 1998). The strains were kindly provided by Prof. M. Forte. Yeast cells were grown at 28 °C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5 to exponential phase (OD546 of 0.6–0.7 OD) or to stationary phase (OD546 of 1.6–1.8). The average doubling times for growth of wild type and Δpor1 cells under these conditions were 3.1 and 4.3 h, respectively. Then H2O2 was added to a final concentration of 1, 3 or 10 mM and the cells were collected after 3 h. The effect of H2O2 on the cell growth rate was assessed by comparison of size of parent and daughter cells under light microscope (Lord and Wheals, 1980). When indicated, minocycline (Sigma-Aldrich) was added in the presence or absence of 10 mM H2O2 and the cells were also collected after 3 h. Cell viability was measured by plating serial dilution of treated and untreated cells on YPG plates and growing up the cells at 28 °C for 4 days.

2.2. Isolation of cell extracts and cytosol and mitochondrial fractions

Cell extracts, cytosol fractions and mitochondria were isolated from pretreated or untreated yeast cells in a given growth phase. Cell extracts were isolated according to O’Brien et al. (2004). Cytosol fraction was isolated from spheroplasts according to the published procedure (Daum et al., 1982; Sturtz et al., 2001). Mitochondrial fraction was isolated by the fast yeast mitochondria preparation protocol. Briefly, cells (100 ml of a culture) were harvested by centrifugation (5 min, 2 500 × g) and washed twice with water. The cell pellet was resuspended in SHK buffer (0.6 M sorbitol, 20 mM Hepes-KOH pH 7.4, 80 mM KCl) containing 1 mM PMSF and the cell suspension was vortexed with glass beads (0.5 mm) four times, for 30 s each time, with 1 min of cooling on ice in between each vortexing step. The suspension was spun down (3 min, 1000 × g) and the obtained supernatant containing mitochondrial fraction was centrifuged (10,000 × g, for 10 min). The pellet of mitochondria was resuspended in SM buffer (250 mM sucrose, 10 mM MOPS-KOH pH 7.2). The integrity of the mitochondrial outer membrane was tested after mitochondria pre-treatment with proteinase K by immunodecoration of Western blots with antisera against yeast marker proteins of the intermembrane space (CC,HL — cytochrome c, heme lyase) and mitochondrial matrix (Mge1p) (Kmita et al., 2003). The calculated mean value of the mitochondrial outer membrane intactness was 95% and 93% for wild type and Δpor1 mitochondria, respectively.

2.3. Determination of the cytosol redox state and levels of mitochondrial proteins

The redox state of the cytosol fraction was determined by calculation of the GSH/total glutathione ratio. The total glutathione (GSH + GSSG) and GSH concentrations (in nmol/mg protein) were determined according to Akerboom and Sies (1981) in the presence of glutathione reductase and glyoxalase I, respectively. Protein concentration was measured by the Bradford method. The levels of mitochondrial proteins in cell extracts and mitochondria were visualized after SDS-PAGE by the ECL method following immunodecoration with anti-yeast proper antisera and quantified by ScanPack3. VDAC, CC,HL and Mge1p antisera were kindly provided by Prof. W. Neupert.

2.4. Calculation of H2O2 and minocycline indexes

To calculate values of the H2O2 index for a given mitochondrial protein, the protein levels quantified for mitochondria fractions and cell extracts isolated from exponentially and stationary growing cells were used to calculate ratios between the control culture and cultures modified by the presence of a given concentration of H2O2. Then, the obtained values were used to calculate ratios between cell extract and mitochondrial fraction for a given growth phase and the applied concentration of H2O2. To calculate values of the minocycline index for a given mitochondrial protein, levels of the protein quantified for mitochondria fractions and cell extracts isolated from exponentially and stationary growing cells were used to calculate ratios between control culture and cultures modified by addition of H2O2 and minocycline added separately or together. Next, the obtained values were used to calculate ratios between cell extract and mitochondrial fraction for a given growth phase and the applied modifications. Finally, the obtained values were used to determine exponential to stationary phase ratios.

3. Results

3.1. Viability of S. cerevisiae cells following their exposure to H2O2 in the presence of VDAC and coincides with changes of mitochondrial protein content

To allow for the effect of H2O2 on S. cerevisiae cells has been analyzed from several perspectives. For example, it is known that H2O2 induces apoptosis in S. cerevisiae as well as in mammalian cells (Magherini et al., 2007). Because proteins involved in the apoptotic mechanism are still a matter of debate (e.g., Kroemer et al., 2007), we decided to check the effect of the presence of VDAC (voltage dependent anion selective channel) on S. cerevisiae cell sensitivity to H2O2. Mitochondria of S. cerevisiae contain only one channel-forming VDAC isoform (VDAC1, termed here VDAC) (Blachly-Dyson et al., 1997: Lee et al., 1998) that simplifies studies on the channel. It has been shown that significant apoptosis of S. cerevisiae cells is obtained by their incubation with up to 10 mM H2O2 (e.g., Darouj et al., 2004; Madeo et al., 1999). Therefore, we used 1, 3 or 10 mM H2O2 for 3 h to induce death of exponentially and stationary growing S. cerevisiae cells in complete glycerol (respiratory-supportive only) containing medium (YPD) compared with the control. The division of S. cerevisiae cells is asymmetrical, giving two cells: the old (parent) and the new (daughter) one that differ in size. As the growth rate decreases, the difference in size becomes more accentuated (Lord and Wheals, 1980). The H2O2 pretreatment up to 10 mM did not change differences in sizes of parent and daughter cells as observed by light microscopy (not shown). On the other hand, wild type cells grow faster at 28 °C in YPG medium than Δpor1 cells (see Materials and Methods), and consequently they form more intense "dots" after spotting onto YPG plates. As shown in Fig. 1A, cell viability, presented as a capability to grow
of serial dilutions of pretreated and untreated cells on YPG plates, depended on the H$_2$O$_2$ concentrations and was distinctly weakened in the case of Δpor1 cells. Moreover, for both strains, cells in exponential growth phase were more sensitive to H$_2$O$_2$ pretreatment than cells in stationary growth phase. At the highest concentrations of H$_2$O$_2$, i.e. 10 mM and for the exponential growth phase, wild type cells growth was observed up to 1:10$^2$ dilution, whereas Δpor1 cells had a significant growth only when undiluted. At the same concentration of H$_2$O$_2$ and for the stationary growth phase, wild type cells growth was observed up to 1:10$^3$ dilution, whereas Δpor1 cells displayed significant growth up to 1:10 dilution. Therefore, under these conditions wild type cells were approximately two orders of magnitude more resistant to H$_2$O$_2$ than Δpor1 cells.

If VDAC is indeed important for _S. cerevisiae_ cell viability after pretreatment with H$_2$O$_2$ the content of VDAC in the pretreated wild type cells should coincide with the applied concentration of H$_2$O$_2$ and the observed capability to grow. To express the correlation, we determined the H$_2$O$_2$ index (see Materials and methods) for VDAC and for Mge1p that we used as a loading control (e.g., Budzinska et al., 2007; Gałganska et al., 2008). The H$_2$O$_2$ index for a given mitochondrial protein denotes a value of a quotient of control level versus modified culture condition level ratios between cell extract and mitochondrial fraction. The yeast colonies illustrate typical results of three independent experiments. Data shown in (B) are mean values ± S.E.M. of five independent experiments. The gels illustrate typical results of these experiments.

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Minocycline is a semi-synthetic tetracycline derivative with postulated neuroprotective potency in the treatment of several neurological diseases including Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis as well as of experimental models of brain injury. The minocycline-mediated neuroprotection is still a matter of debate (e.g., Giesel et al., 2009). At the basis of obtained data we have suggested that minocycline might be regarded as VDAC modulator that favors VDAC closing and affecting VDAC regulatory proteins (Garcia-Martin et al., 2010). Therefore, we decided to check the protective effect of minocycline on wild type and Δpor1 cells exposed to 10 mM H$_2$O$_2$. First, we determined the most effective concentration of minocycline using exponential growth phase wild type cells because of their sensitivity to H$_2$O$_2$ pretreatment (Fig. 1). As shown in Fig. 2A, increasing concentrations of minocycline (10, 50 and 100 μM) caused a gradual increase in capability of the cells pretreated with 10 mM H$_2$O$_2$ to grow on YPG plates with the highest increase observed in the presence of 100 μM minocycline. Then, we checked the effect of 100 μM minocycline on exponentially and stationary growing wild type and Δpor1 cells pretreated with 10 mM H$_2$O$_2$. As shown in Fig. 2B, minocycline itself in the applied concentration did not change capability of the studied cells to grow on YPG plates and its cytoprotective effect was clearly visible only for exponential growth.

![Graph](image_url)
phase wild type cells. On the other hand, independently of the studied growth phase, Δpor1 cells were not amenable to minocycline.

To quantify the effect of minocycline for wild type cells, we calculated the minocycline index (see Materials and methods) for VDAC and Mge1p. To obtain the minocycline index, a quotient of control level versus modified culture condition level ratios between cell extract and mitochondrial fraction were calculated for a given mitochondrial protein and used to determine exponential to stationary phase ratios. Thus, the minocycline index reflects differences in changes of VDAC and Mge1p content between exponential and stationary phases resulting from changes of mitochondria mass (cell extracts) and changes of expression levels in mitochondria. The changes were induced by the cell pretreatment with 100 μM minocycline and 10 mM H2O2 applied separately or together. As shown in Fig. 2C, minocycline did not change content of VDAC and Mge1p as the calculated value of the minocycline index was close to 1. However, when added together with H2O2, minocycline distinctly weakened the effect of H2O2 on the studied protein content that coincided with the observed enhancement of cell viability. Interestingly, the increase in the minocycline index was slightly more pronounced for VDAC, namely the value of the index increased from 0.3 ± 0.07 to 0.74 ± 0.04 for VDAC and from 0.40 ± 0.04 to 0.78 ± 0.07 for Mge1p.

3.3. The protective effect of minocycline against hydrogen peroxide in S. cerevisiae cells coincides with the cytosol redox state

It has been reported that the permeability of the plasma membrane and the cell wall to H2O2 decreases during stationary growth phase (Maris et al., 2001; Sousa-Lopes et al., 2004). Accordingly, we observed that for both strains, the cells in exponential growth phase were more sensitive to H2O2 pretreatment than those in stationary growth phase (Fig. 1). Therefore, we decided to check the effect of the pretreatment with H2O2 and/or minocycline on the cytosol redox state of exponentially and stationary growing wild type and Δpor1 cells. As shown in Fig. 3, increasing concentrations of H2O2 and 100 μM minocycline added separately or together with 10 mM H2O2 caused distinct changes of the cytosol redox state only in the case of exponentially growing wild type cells. Under the same conditions, exponentially growing Δpor1 cells displayed nearly stable levels of the cytosol redox state. On the other hand, stationary growing wild type and Δpor1 cells pretreated with H2O2 and with minocycline added separately or together with H2O2 did not exhibit pronounced changes of the cytosol redox states. Moreover, for the both strains the calculated values of coefficient of change of the cytosol redox state were comparable and close to 1 that denotes weak changes.

4. Discussion

We have shown that VDAC (voltage dependent anion channel) contributes to viability of S. cerevisiae cells following their exposure to H2O2. Mitochondria of S. cerevisiae express two VDAC isoforms, of which only one has been proved to form a channel (VDAC1, encoded by the POR1 gene (Blachly-Dyson et al., 1997: Lee et al., 1998) and termed here VDAC) that facilitates studies on the channel. The viability of the isogenic wild type and Δpor1 cells pretreated with H2O2 on YPG plates, shown as a capability to grow of their serial dilution (Fig. 1A), depends on the H2O2 concentrations and is distinctly weakened in the case of Δpor1 cells. Interestingly, it has been reported that the activity of catalase is distinctly changed in Δpor1 cells when compared to wild type cells (Galgańska et al., 2008) that may be a part of involved mechanism. Moreover, independently of the studied strain, cells in exponential growth phase are more sensitive to H2O2 pretreatment than cells in stationary growth phase. The last observation is in agreement with published data and is explained by a decreased permeability of the plasma membrane and the cell wall to H2O2 during stationary growth phase (Maris et al., 2001; Sousa-Lopes et al., 2004). Interestingly, the content of the studied mitochondrial proteins in the pretreated wild type cells, as shown by the H2O2 index values (Fig. 1B), coincides with the applied concentration of H2O2 and the observed capability to grow. The index reflects changes of a given protein content resulting from changes of mitochondria mass (cell extracts) and changes of expression levels in mitochondria.
Taking into account changes of the index values calculated for VDAC and Mge1p, used as a loading control (e.g., Budzinska et al., 2007; Galganska et al., 2008), one could conclude that the observed capability of S. cerevisiae cells to grow following pretreatment with \( \text{H}_2\text{O}_2 \) coincides with content of the studied mitochondrial proteins, particularly in exponential phase when the cells are more sensitive to \( \text{H}_2\text{O}_2 \) pretreatment. On the other hand, changes concerning VDAC are more pronounced than those of Mge1p that suggests more significant role of VDAC in cell viability. Accordingly, it has been observed for animal cells that \( \text{H}_2\text{O}_2 \)-induced apoptosis also coincides with expression levels of VDAC (Gupta et al., 2007; Xiong et al., 2009). However, it is unclear how VDAC contributes to S. cerevisiae cell resistance to \( \text{H}_2\text{O}_2 \) pretreatment. It is noteworthy that reconstituted VDAC was not affected by \( \text{H}_2\text{O}_2 \) up to concentration of 10 mM (not shown). The published data indicate that sensitivity of S. cerevisiae cells to \( \text{H}_2\text{O}_2 \) depends on mitochondrial function (e.g., Grant et al., 1997; Thorpe et al., 2004). It is suggested that the \( \text{H}_2\text{O}_2 \) sensitivity is due to a defect in an energy-requiring process that is needed for detoxification of \( \text{H}_2\text{O}_2 \) or for the repair of oxidatively damaged molecules. It is in agreement with the observation that depletion of VDAC distinctly affects the metabolite passage across the outer membrane of S. cerevisiae mitochondria and consequently the efficiency of the respiratory chain and adenine nucleotide exchange (Kmita et al., 1999; Lee et al., 1998) that are regarded to be significant for the defense against \( \text{H}_2\text{O}_2 \). Thus, it could be speculated that altered functioning of mitochondria that occurred in the absence of VDAC handicaps the involved protecting mechanisms. On the other hand, it cannot be excluded that within \( \text{H}_2\text{O}_2 \)-pretreated cells reduced transition metal ions would cause formation of hydroxyl radical from \( \text{H}_2\text{O}_2 \) (Stohs and Bagchi, 1995) that could cause VDAC oxidative damage as VDAC is known to be highly sensitive to this kind of damage (O’Brien et al., 2004). This, in turn, would result in wild type cell death, especially under condition of an increased access of \( \text{H}_2\text{O}_2 \), i.e., during the exponential growth phase.

The increased viability of exponentially growing cells containing VDAC is potentiated by minocycline, an antibiotic of the tetracycline family that displays cytoprotective potency (Garcia-Martinez et al., 2003). Minocycline itself does not change the capability of the cells to grow on YPG plates (Fig. 2A, B), but its cytoprotective effect is clearly visible only for exponentially growing wild type cells. Thus, the protective effect of minocycline on S. cerevisiae cells depends on their sensitivity to \( \text{H}_2\text{O}_2 \) pretreatment and the presence of VDAC. To quantify the effect of minocycline for wild type cells we calculated the minocycline index for VDAC and Mge1p. The index reflects differences in changes of VDAC and Mge1p content between exponential and stationary phases resulting from changes of mitochondria mass (cell extracts) and changes of expression levels in mitochondria (Fig. 2C). Taking into account the values of the minocycline index one could conclude that minocycline does not change content of VDAC and Mge1p but when added together with \( \text{H}_2\text{O}_2 \), minocycline distinctly weakens the effect of \( \text{H}_2\text{O}_2 \) on the studied protein content that coincides with the observed enhancement of cell viability. As the increase in the minocycline index value is slightly more pronounced for VDAC, it can be suggested that in S. cerevisiae cells VDAC is important for sensitivity to \( \text{H}_2\text{O}_2 \) and protective effect of minocycline against \( \text{H}_2\text{O}_2 \). It has been reported (e.g., Vander Heiden et al., 2001) that pro-apoptotic signals can maintain metabolite exchange across the outer mitochondrial membrane by inhibiting VDAC closure. On the other hand, we have observed (Garcia-Martinez et al., 2010) that minocycline added to a reconstituted system causes VDAC transition into lower conductance states, decreases the voltage dependence in a concentration-dependent way and probably causes VDAC transient blockade/closure. Thus, in the presence of minocycline, the permeability of the mitochondrial outer membrane mediated by VDAC might be affected, which contributes to cell protection. It is well known that VDAC closing is accompanied by a change of its selectivity toward cations and the channel never closes totally (e.g., Benz, 1994; Colombini et al., 1996). Thus, VDAC adopts a fully open state and multiple closed states that differ in permeability. However, it has been also proven that VDAC has a cation selective open state (Pavlov et al., 2005). Therefore, Mannella and Kinnelly (2008) have proposed that VDAC modulators triggering a transition to lower conductance states might be acting not to close the channel but to open it.

The protective effect of minocycline also coincides with distinct changes of the cytosol redox state (Fig. 3). \( \text{H}_2\text{O}_2 \) and minocycline added separately or together cause clear changes of the cytosol redox state only in the case of exponentially growing wild type cells. Under the same conditions, exponentially growing \( \Delta \text{por1} \) cells displayed nearly stable levels of the cytosol redox state. Stationary growing wild type and \( \Delta \text{por1} \) cells pretreated with \( \text{H}_2\text{O}_2 \) and/or with minocycline do not display distinct changes of the cytosol redox states. Accordingly, glutathione (GSH) appears to be an important antioxidant for protection of S. cerevisiae cells against \( \text{H}_2\text{O}_2 \) (Grant et al., 1998; Poljak et al., 2003). Interestingly, VDAC influences the reduction levels of glutathione (Budzinska et al., 2007; Galganska et al., 2008). Moreover it is proposed that VDAC participates in reduction/oxidation mechanism important for communication between mitochondria and the nucleus (Galganska et al., 2010). The obtained results indicate that the ability to change the cytosol redox state following exposure to \( \text{H}_2\text{O}_2 \) or/and minocycline appears to be an intrinsic feature of exponentially growing cells (young cells) containing VDAC. Moreover, the ability seems to be crucial for both cell viability and protective effect of minocycline.

In summary, the present work has revealed for the first time the protective effect of VDAC against \( \text{H}_2\text{O}_2 \) in exponentially growing S. cerevisiae cells and the involvement of the protein in cytoprotective...
activity of minocycline. Thus, under conditions of increased accessibility of H$_2$O$_2$ to S. cerevisiae cells VDAC seems to be an important part of the cell defense system.

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