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Short communication

Biochemical study of idebenone effect on mitochondrial metabolism of yeast

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Abstract

The aim of this work was to study the effect of the drug idebenone on the growth of a strain of *Saccharomyces cerevisiae* yeast and its respiratory-deficient mutant (rho^0). We took this yeast as a model system of the interaction of the drug with mammalian cells. The effect of idebenone was evaluated in rich and minimal media. In the S288c strain, idebenone exerted a growth inhibitory effect in concentrations higher than 50 μM in media containing a carbon source consumed at mitochondrial level. In conditions of low oxygen supply, idebenone allows yeast to keep a cellular yielding comparable with conditions of normal oxygen supply. Also, the presence of idebenone in the growth media increased by 50% the fluorescence signal of rhodamine 123, indicating a higher mitochondrial membrane potential. The results could explain the effect of idebenone in the treatment of diseases in which oxygen deficiency alters the energetic metabolism of the cell.

Keywords: Idebenone; *Saccharomyces cerevisiae*; Mitochondrial potential

1. Introduction

The biochemical study in a microorganism like the yeast *Saccharomyces cerevisiae* allows the study and comprehension of the function of genes implicated in human diseases (Oliver, 2002; Steinmetz et al., 2002; Berger et al., 2005; Vacher et al., 2005; Calvo et al., 2006). Also, it is possible to analyze the effect of drugs in metabolic pathways common to mammalian and yeast cells (Fetchko et al., 2003; Geesaman, 2006).

One of these alterations is related with the deficiency in oxygen supply to the tissues. It is well established that ischemic situations or hypoxemia could lead to different injuries at cellular level (Galaris et al., 2006; Ma et al., 2006; Oechmichen and Meissner, 2006). On the other hand, there are situations, such as sleep apnea, in which the period of oxygen absence is followed by a normal supply of oxygen (Lavie, 2003; Lavie et al., 2004).

Due to the fact that yeast cells can grow in both aerobic and anaerobic conditions, the system looks potentially valuable to study growth effects and cellular damage associated with conditions of low oxygen supply (Bunn and Poyton, 1996).

Idebenone is a synthetic analog of coenzyme Q (Esposito et al., 1996; Geromel et al., 2002). Coenzyme Q (CoQ) is an important membrane antioxidant and essential to the mitochondrial electron transport chain. The idebenone structural similarity with CoQ allows the compound to be used in the therapy of several diseases to increase mitochondrial metabolism.

Such is the case in the treatment of patients with alzheimer, hepatic oxidative stress, brain-vascular diseases and Friedreich's ataxia (Gutzmann and Hadler, 1998; Thal et al., 2003; Schols et al., 2004). Due to the capacity of idebenone to prevent lipid peroxidation, the drug protects mitochondrial membrane integrity toward oxidative damage (Genova et al., 2003). Its antioxidant properties, concurrently with the fast absorption, allow the compound to maintain cellular structures in conditions of brain ischemia and injuries in the central nervous

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system (Esposito et al., 1996; Geromel et al., 2002). Also, its interaction with the electron transport chain preserves the ATP synthesis in ischemic studies.

In our laboratory, we have previously used yeast as a model system to establish the effect of L-carnitine on the growth inhibition caused by iron (Stella et al., 2005) in the microorganism. In diseases such as Friedreich's ataxia and Parkinson, an increase on the mitochondrial iron concentration level that leads to an increase in the synthesis of free radicals has been described (Seznec et al., 2004).

Based on our previous experience, the aim of the present work was to expand the knowledge of the interaction of idebenone with the microorganism's metabolism as a model system of eucaryotic cells. The work was performed with a wild type strain and its respiratory-deficient mutant (rho^0). In addition, we evaluated whether the system produces results comparable to those obtained with other model systems (Walberg, 2000).

2. Materials and Methods

2.1. Strains

The yeast strain used in the experiments was the wild type strain S288c. The respiratory-deficient strain was obtained from the wild type by using the ethidium bromide treatment (Ausubel et al., 1999).

2.2. Growth media

Inocula of S288c were prepared in YPD medium (1% yeast extract, 1% peptone, 2% D-glucose). For the assays in liquid medium, the cells were grown in the following media: YPD (1% yeast extract, 1% peptone, 2% D-glucose), YPG (1% yeast extract, 1% peptone, 3% glycerol) and CAV medium (0.5% casaminoacids, 0.1% ascorbic acid, vitamins, Kotliar et al., 1990) and 2% glucose or 3% glycerol as the carbon source. For the VJ medium (Vavra and Johnson, 1956), ammonium ions were used as the nitrogen source (3 g/l), while 0.05% glucose and 3% glycerol were the carbon source. The cells were grown at 30 °C with constant agitation. In assays where the effect of idebenone was evaluated on solid YPD medium, the detergent SDS was added to a final concentration of 5×10^{-3}%.

2.3. Inhibition in liquid medium

Inhibition in liquid medium was evaluated by optical density (OD) changes of the culture measured at 570 nm or by colony counting in solid medium. The initial value of OD_{570} of the cultures was 0.060 (1.5×10^6 cells/ml). Idebenone was added from a 17.6 μM solution in DMSO.

2.4. Yeast growth in solid medium with top agar

Plates holding CAV medium with agar 2% were inoculated with 12 ml of a 1% agar suspension carrying approximately 500 CFU of the S288c strain.

2.5. Rhodamine 123 (Rh123) spectra

Cells (1.0 ml) grown into the selected conditions were suspended at a concentration of 2×10^8 cells/ml in HEPES 10 mM, pH 6.8. After the addition of Rh123 to a final concentration of 10 mM the mixture was incubated at 30 °C for 30 min. Following incubation, cells were centrifuged and the precipitate washed with HEPES 10 mM. Then, cells were resuspended at a final volume of 1.0 ml. An aliquot of 25 μl or 50 μl was diluted in 1.8 ml of HEPES to achieve the fluorescence spectra (Ex = 485 nm) in a Jasco FP-770 equipment.

3. Results

3.1. Effect of idebenone in media with different carbon sources

S. cerevisiae cells can grow in media with a carbon source that requires a fermentative or aerobic metabolism. In our case, we chose D-glucose 2% for the first condition and glycerol 3% for the second one. The cells of the S288c strain were inoculated at an initial density of 1.5×10^6 CFU/ml in the selected medium. The idebenone was added immediately, and the suspensions were grown at 30 °C and constant agitation. At 24 and 48 h of growth, aliquots were separated to determine the values of optical density. Also, from the same aliquots, dilutions were made to inoculate YPD plates for colony counting. Fig. 1 shows the obtained results of OD_{570} after 48 h of growth. No changes were observed when cells were grown in glucose media (YPD), but in glycerol media (YPG) idebenone inhibited the growth beginning at 50 μM, reaching 72% of growth at 75 μM when compared to the control value.

This result can be explained at least in two ways. It is known that glycerol as a carbon source produces a cell with a thinner wall. As a result of a thinner permeability barrier, idebenone could be taken easily into the cell interior, where at high concentrations, it would generate a harmful effect for cellular metabolism. Also it is possible that idebenone requires for its interaction a mitochondria which is carrying out the main energy supply to the cell, through oxidative phosphorylation.

In a previous work we showed that the addition of SDS detergent to the culture medium facilitates the diffusion of drugs to the intracellular media (Pannunzio et al., 2004). In a similar way to the previous experiments, YPD media were supplemented with the addition of detergent at 5.0×10^{-3}% final concentration. This detergent concentration caused less than 10% decrease in cellular viability. Then the YPD media were inoculated with either the wild type strain or the respiratory-deficient

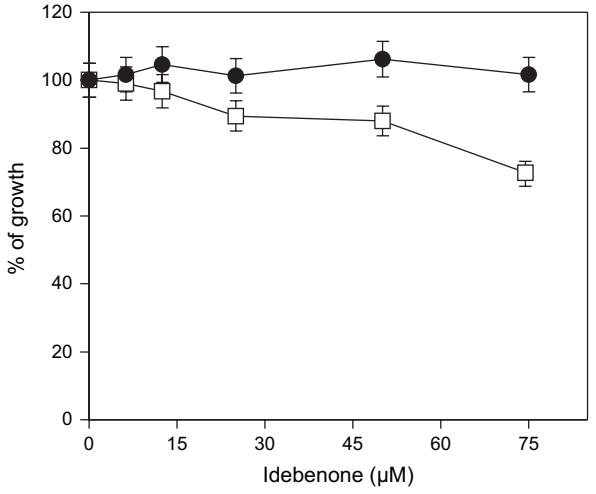

Fig. 1. S288c cells grown for 48 h. An aliquot of each medium was separated for colony counting. The medium with glucose (●) as the carbon source rose to a cellular yielding of 1.0×10^8 cells/ml while the growth with glycerol (□) gave 3.8×10^7 cells/ml.

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Table 1
Yeast growth on liquid YPD in idebenone presence and SDS addition

Idebenone (μM)	OD_{570} (% of control) in S288c	OD_{570} (% of control) in rho^0
0	10.40 (100)	6.00 (100)
44	9.92 (95.4)	5.70 (95)
64	9.51 (91.4)	5.49 (91.5)
88	6.70 (64.4)	5.22 (87.0)

Yeast cells were grown in YPD medium supplemented with SDS to a final concentration of 5×10^{-3}%. The rho^0 cells were prepared by ethidium bromide treatment in order to obtain cells without mitochondrial respiration.

mutant (rho^0) obtained by the ethidium bromide treatment. Table 1 shows the values of idebenone obtained at 44, 64 and 88 μM. The wild strain reached 65% of the control value, while the respiratory-deficient strain reached almost 83% of the control value.

The absence of effect of idebenone in the YPD media could be explained by the drug incapacity to go across the permeability barrier. Then, SDS addition would modify the permeability, allowing the drug to move into the cell. The difference in the extent of the observed inhibition between the wild strain and the rho^0 mutant could be explained by the fact that mitochondrial activity is the main target of the drug.

Synthetic media have the advantage of having a definite chemical composition that allows to link each component of the medium with the observed effect or phenotype. Considering that standard synthetic medium contains only the ammonium ion as the nitrogen source we considered other sources to supply the nitrogen requirement. On the base of the Potato-dextrose medium for yeasts (Difco Laboratories, 1953), we prepared a medium with amino acids as the nitrogen source and supplemented it with vitamins and ascorbate (CAV).

Similarly to the growth in YPD or YPG, the effect of idebenone was assayed in CAV medium with glucose or glycerol as the carbon source. Results obtained with glycerol showed a growth inhibition starting at 50 μM concentration. Differently, when glucose was used as a carbon source, we found 25% increase in cellular yield when compared with the medium without the drug (data not shown).

3.2. Effect of idebenone on growth under limited oxygen levels

In yeast cells there are many experimental protocols for growth in conditions of oxygen limitation. A simple procedure (Difco Laboratories, 1953) is to pour a top agar suspension into the inoculated plate. In our laboratory, we assayed S288c growth in YPG medium either with or without the top agar addition. We observed (data not shown) that in this medium the presence of top agar reduced the yielding of cells, estimated by the number and the diameter of the colonies. This reduction was not observed in the YPD medium since the presence of glucose shifts the cells toward a fermentative metabolism.

In this condition, we assayed the effect of idebenone by using CAV medium either with or without 1% a top agar layer after the cells inoculation. In the absence of idebenone and top agar, the number of colonies obtained was almost 50% higher than the colonies obtained in the medium with the top agar addition (Table 2). Differently, with idebenone and keeping this limited oxygen conditions, the number of colonies increased.

Table 2
Effect of idebenone on the growth of S288c on solid YPD

Idebenone (μM)	Top agar	Number of colonies
0	–	249 ± 12
0	✓	103 ± 7
44	–	335 ± 14
44	✓	392 ± 17

CAV medium with glycerol as the carbon source prepared with 2% agar was used to prepare plates to support the growth of the colonies. To inoculate the medium, 12.0 ml of a 1% top agar suspension with about 500 CFU of the yeast was poured into this medium. This last suspension was supplemented with idebenone from a 17.6 mM solution in DMSO or only with DMSO for the controls.

3.3. Effect of idebenone on rhodamine 123 (Rh123) lipophilic cation accumulation

Rhodamine 123 is a lipophilic compound that accumulates inside mitochondria, which could be used to analyze, at least qualitatively, the value of mitochondrial membrane potential. Therefore, from our previous assays in YPG media, we chose this medium to test the effect of idebenone on rhodamine 123 accumulation. We used the VJ medium since its defined chemical composition eliminates effects due to yeast extract and peptone compounds. The medium was inoculated with the wild strain and the cells were grown for 48 h. The cells were incubated with Rh123 according to the previously described protocol. The spectra obtained showed that idebenone increased the fluorescence signal and this fact can be connected with a greater load of the dye by the organelle (Fig. 2).

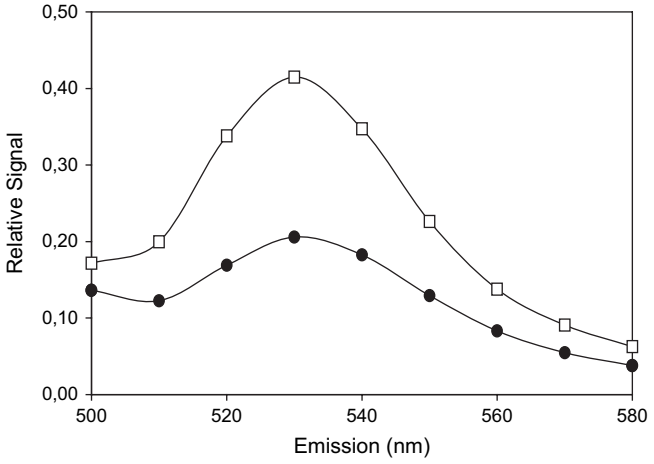

Fig. 2. Cells (1.0 ml) from a control medium (●) or with idebenone addition (□) were suspended at a concentration of 2×10^8 cells/ml in HEPES 10 mM, pH 6.8. After the addition of Rh123 to a final concentration of 10 mM the mixture was incubated at 30 °C for 30 min. The fluorescence spectra were performed with an Ex = 485 nm in a Jasco FP-770 equipment.

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4. Discussion

Results obtained using yeast as a model system showed that idebenone has an effect on the microorganism growth, which depends on different culture conditions.

In culture media with a carbon source such as glycerol, which is metabolized at a mitochondrial level, a higher level of inhibition was noticed.

Glycerol produced a thinner cell wall that could lead to a more permeable barrier for the compound, but also demanded the cell to obtain its energy mainly from mitochondrial metabolism. This condition may produce a cell with a higher level of free radicals from the organelle activity. In connection with this, it has also been observed that idebenone and other quinones inhibit *Helicobacter pylori* growth by inhibiting respiration and decreasing the ATP level (Sakiko et al., 2006).

Moreover, in cortical astrocytes of rats with 50 μM concentration (Paolino et al., 2004) a toxic effect of idebenone due to a cellular membrane perturbation has been observed. This last concentration is the same as the one that showed inhibitory effects in our system.

However, the mechanism in *S. cerevisiae* may be different from the one of *H. pylori* since at 50 μM idebenone has shown a protective effect from the strong inhibitory effect over the cell growth exerted by menadione at 25 μM (unpublished results). Menadione is a quinone that increases the level of radical oxygen species (ROS) on yeast.

The fact that idebenone in YPD medium supplemented with SDS inhibited yeast growth shows that the drug interaction does not require mitochondria working at their top oxidative level. Furthermore, the system showed a greater inhibition in the wild strain than in the respiratory-deficient strain, thus confirming that this organelle is the main target of the drug. The characteristic of the yeast respiratory-deficient strain will permit future works to establish other biological effects in cells without functional mitochondria. It is known that besides the rho^0 mutants, it is possible to prepare yeast mutants where the cell has a partial deficiency in the mitochondrial function, such as the deficiency in the ADP/ATP mitochondrial translocator.

Results obtained in our experimental design when top agar was present, a condition that limits the oxygen contribution to the cell, indicate that idebenone increases cellular growth. The effect of the drug in low oxygen conditions gives consistent reasons for considering its use in diseases associated with low oxygen supply, such as chronic apnea, hypoxia or alzheimer's disease. However, at higher concentrations growth inhibition was observed.

It is worth mentioning that the addition of idebenone increased the rhodamine 123 signal, indicating a higher electrical charge or mitochondrial membrane potential. Since the assay was qualitative, it is possible that, in cells with an impaired electron chain or decreased oxygen levels, the presence of idebenone could increase the chain activity to the extent of a normal cell.

Our present work was aimed at establishing the effect of the drug in different growing conditions and also in respiratory mutants. Since the microorganism molecular biology is simple and many of its genes are homologous to mammals, this model system would contribute and amplify the knowledge on the subject.

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