Bax expression protects yeast plasma membrane against ethanol-induced permeabilization

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Abstract The mechanism by which the expression of pro-apoptotic protein Bax is able to kill yeast was investigated. Ethanol stress induces a permeabilization of the plasma membrane revealed by propidium iodide accumulation. Bax expression, although killing yeast cells, prevents this permeabilization. These effects are modulated by aeration, by manipulation of the unsaturation index of fatty acids and by addition of resveratrol, a known inhibitor of lipid oxidation. These data suggest that lipid oxidation is involved in Bax effects. Taken together, these data show for the first time a direct effect of Bax on plasma membrane permeability properties and suggest that yeast is a powerful tool for investigating the molecular mechanisms underlying this process. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ethanol stress; Bax; Flow cytometry; Resveratrol; Yeast; Lipid oxidation

1. Introduction

Apoptosis is an active form of cell death essential for the development and homeostasis of multicellular organisms. Until recently, unicellular eukaryotes, like the yeast *Saccharomyces cerevisiae*, seemed to be devoid of such a program. However, the heterologous expression of known mammalian apoptotic regulators like pro-apoptotic Bcl-2 family members (see [1,2] for reviews) or caspases [3] induces apoptosis-like characteristics in yeast cells. In addition, some mutants [4–6], oxidative stress [5] as well as the ageing process [7] induce the appearance of similar characteristics as the heterologous expression of mammalian apoptosis regulators [5,8]. These observations support the idea that some form of programmed cell death exists in *S. cerevisiae*, although the physiological function of such a program remains unclear [2,9].

Induction of apoptotic characteristics in yeast has often been achieved by the heterologous expression of the pro-apoptotic protein Bax. Like in mammalian cells, Bax is addressed and inserted in the outer mitochondrial membrane where it participates in the formation of a giant channel, termed mitochondrial apoptosis-induced channel [10,11], and induces the release of cytochrome *c* [12]. However, this release of cytochrome *c* is not likely to participate directly in yeast cell death since Bax expression in a cytochrome *c*-less strain still induced cell death, although with slower kinetics [13]. Other characteristics observed in Bax-expressing yeast are nuclear DNA fragmentation and phosphatidylserine exposure on the outer leaflet of the plasma membrane [5,6]. One of the main characteristics of apoptosis, as compared to other forms of death, such as necrosis, is the late maintenance of the permeability barrier of the plasma membrane, which normally prevents the release of intracellular components on neighboring cells and the subsequent inflammatory reaction. The molecular mechanisms involved in this maintenance and a possible direct role of pro-apoptotic Bcl-2 family members, such as Bax, are not elucidated.

In the present paper, we studied the characteristics of death induced by ethanol, which is a common stress occurring in yeast growing under fermentative conditions (see [14] for review). Ethanol-induced cell death is accompanied by a loss of the plasma membrane permeability barrier. Importantly, we show that Bax expression triggers ethanol-induced cell death towards a form of death, accompanied by a late maintenance of plasma membrane permeability barrier. These results strongly support the idea that yeast has the endogenous components allowing cell death without plasma membrane permeabilization, and that heterologous Bax expression is able to activate these components. Experiments are reported supporting the hypothesis that lipid oxidation is involved in these effects.

2. Materials and methods

2.1. Yeast strain, culture media and conditions

The haploid wild-type strain W303-1A (*matα*, *ade1*, *his3*, *leu2*, *trp1*, *ura3*, *can*^{R}) was used in all the experiments. The construction of pCM189-Bax plasmid has been described previously [13]. This plasmid allows controlled expression of c-*myc*-tagged Bax under the control of a *tet*-off promoter. Bax-c-*myc* is constitutively active in yeast, and does not require further activation. Cells are usually grown under repressive conditions, in the presence of 10 μg/ml doxycycline (Sigma). Bax expression was induced by three washes of the cells in water and resuspension in a medium without doxycycline. Culture media contained 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate, 0.1% potassium phosphate, 0.2% Drop-Mix and, depending on the experiments, 0.5% D-glucose, 6% D-glucose, 6% D-galactose or 2% DL-lactate. The pH was adjusted to 5.5 with NaOH and HCl. Some

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experiments were done with a homemade minimal medium, according to [15]. Cells were batch-grown aerobically at 28°C, in 100-ml Erlenmeyer flasks containing 10 ml of medium, under rotary shaking (200 rpm). Growth was measured as the turbidity of the cell suspension at 550 nm.

2.2. Cell death measurements

Aliquots of cultures containing 200 cells were plated on a complete YPD medium (1% yeast extract, 1% Bacto-peptone, 2% glucose, 2.5% agar) added with 10 μg/ml doxycycline. The number of colonies was counted after 48 h incubation at 28°C.

2.3. Propidium iodide (PI) permeability

Aliquots of cells (about 10^6 cells/ml) were added to the same volume of a solution of PI (100 μg/ml in 50 mM sodium citrate) and incubated in the dark for 30 min at 4°C. Samples were diluted 10 times in 50 mM sodium citrate and PI fluorescence was measured in a Partec Galaxy flow cytometer in the FL3 channel. Each analysis was done on 30 000 cells, counted at a flow rate of 1.5 μl/s.

2.4. Other methods

Ethanol was measured in the culture medium, after centrifugation of the cells, with an ethanol dosing kit (Boehringer) according to the manufacturer's instructions. Whole cell protein extracts were obtained by adding 0.05 ml of 2 M NaOH, 2% SDS to 0.5 ml of cells (10^7 cells/ml); after 15 min incubation on ice, proteins were precipitated by adding 0.05 ml of 3 M trichloroacetic acid. After 15 min incubation on ice and 3 min centrifugation (12 000 × *g*), the pellet was resolubilized and the proteins were separated by SDS–PAGE. Bax expression was controlled by Western blot analysis, using a primary polyclonal anti-Bax antibody (N20, Santa Cruz), a secondary anti-rabbit horseradish peroxidase-labeled antibody (Jackson Laboratories) and ECL detection (Amersham).

3. Results

3.1. Ethanol-induced cell death is accompanied by plasma membrane permeabilization

Cells from the wild-type haploid strain W303-1A were grown aerobically in a liquid medium containing 6% or 0.5% glucose, 6% galactose or 2% lactate, and assayed at different times for plasma membrane permeability to PI. Cultures grown in the presence of 0.5% glucose, 6% galactose or 2% lactate never exhibited a proportion of PI-permeable cells above 10% (Fig. 1). On the other hand, cultures grown in the presence of 6% glucose contained a high proportion of PI-permeable cells (Fig. 1). This proportion of PI-permeable cells increased with time: about 30% after 24 h and up to 50% and 65% after 48 and 72 h, respectively (Fig. 2).

Cell death was quantified simultaneously by plating the cells on a complete YPD medium (2% glucose) and measuring the colony-forming efficiency, showing a good correlation between cell death and PI permeability (Fig. 3).

Since PI permeability and cell death only appeared in cultures grown on a high-glucose medium, under conditions of catabolic repression of respiratory enzymes and further ethanol accumulation, we hypothesized that it was related to etha-

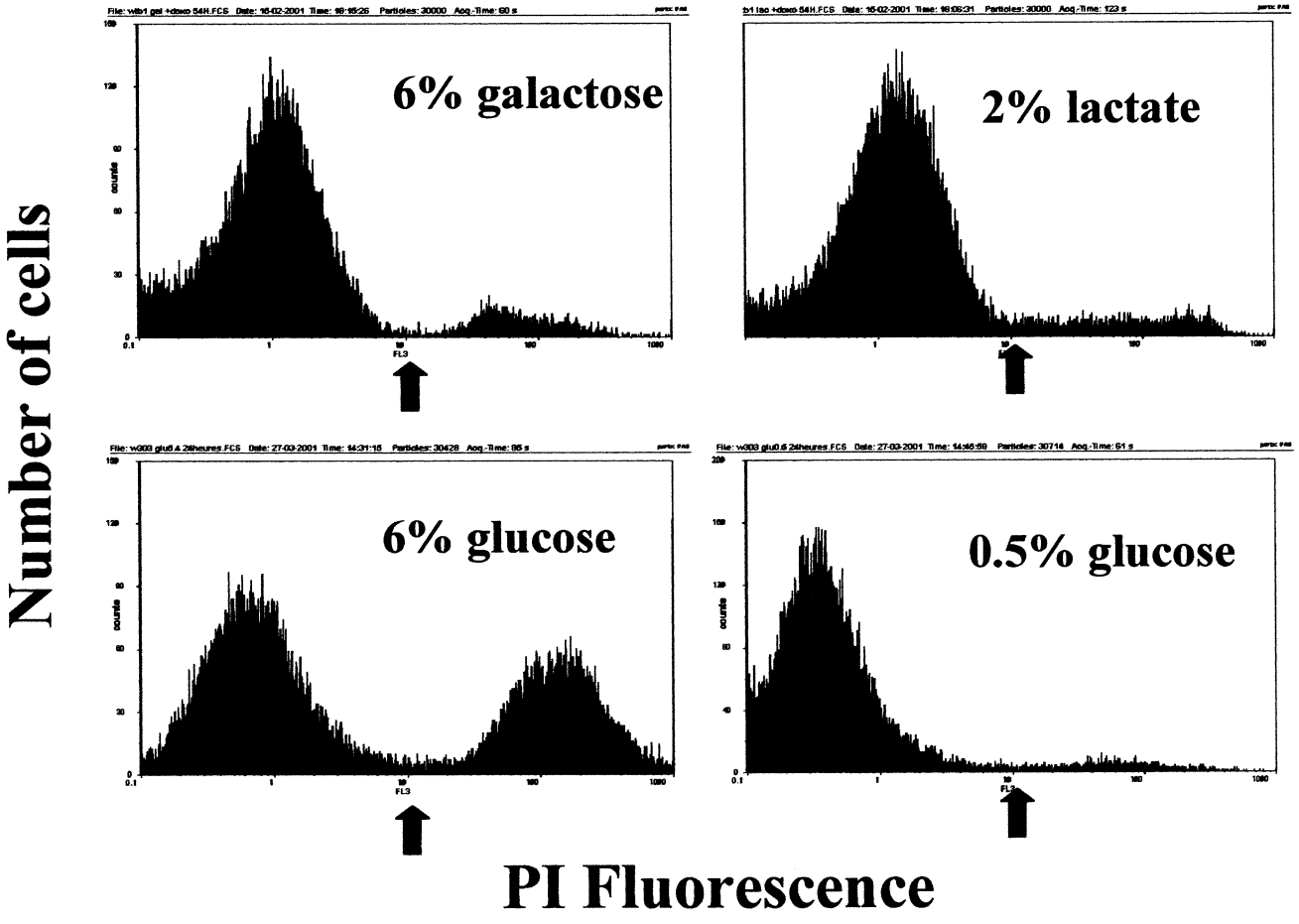


Fig. 1. PI permeability of wild-type strain grown on different carbon sources. Strain W303-1A was grown aerobically in YNB liquid medium supplemented with different carbon sources and PI permeability was measured as indicated in Section 2. The black arrow indicates the fluorescence value above which cells are considered to be PI-permeable.

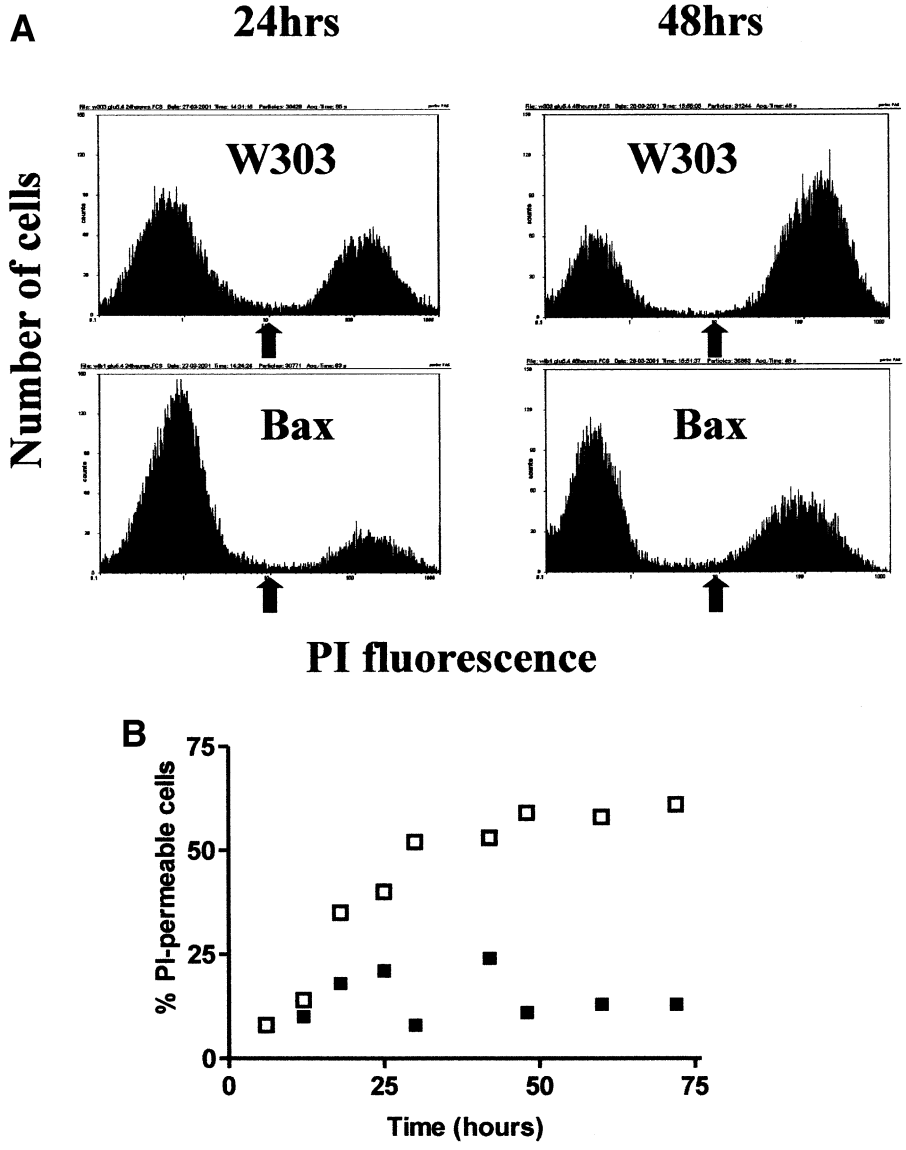


Fig. 2. Bax expression prevents the appearance of PI permeability in 6% glucose-grown cultures. Top: Flow cytometry profile of wild-type (W303) or Bax-expressing (Bax) cultures in 6% glucose after 24 or 48 h. Bottom: The proportion of PI-permeable cells (above the arrow on cytometry profiles) was plotted versus time. (□) W303-1A; (■) Bax-expressing cells.

nol accumulation in the medium. The proportion of cells exhibiting PI permeability was plotted versus the ethanol concentration in the medium: the permeability strongly increased when ethanol reaches a concentration above 15 g/l (Fig. 4A). Direct addition of ethanol to cultures grown in the presence of 0.5% glucose also increased PI permeability and cell death, although to a lesser extent than direct cultures in the presence of 6% glucose (Fig. 4B) suggesting that other parameters in addition to the ethanol concentration might be involved in the appearance of plasma membrane permeability.

3.2. *Bax expression increases cell death but protects against ethanol-induced plasma membrane permeabilization*

PI permeability was measured on the wild-type strain carrying the plasmid pCM189-Bax, which allows the inducible expression of Bax. In the presence of 6% glucose, with this system of expression, the amount of Bax protein detected by Western blot is similar to that in the presence of 2% lactate, as shown previously [13]. The proportion of cells exhibiting PI permeability was halved by Bax expression, reaching 10%, 20% and 30% after 24, 48 and 72 h respectively (Fig. 2).

Cell death was quantified under these conditions: Bax expression induces 50% and $>80\%$ cell death after 48 and 72 h, far above the proportion of PI-permeable cells (Fig. 3).

An obvious explanation for this protective effect could be that ethanol was not accumulated in Bax-expressing cultures, because of the high ratio of cell death. Therefore, ethanol concentration was measured in Bax-expressing cultures. Ethanol was accumulated at similar levels as in control conditions, but no PI permeability appeared when ethanol reached a concentration of 15 g/l (Fig. 4A). Moreover, direct addition of ethanol to cells grown in the presence of 0.5% glucose did not increase PI permeability in Bax-expressing cells (Fig. 4B). These experiments show that the altered response of Bax-ex-

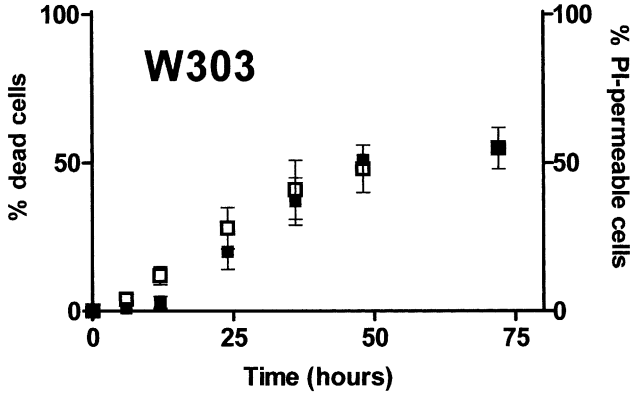
pressing cells is not related to a decrease of ethanol accumulation but to actual changes of the intrinsic properties of the plasma membrane.

3.3. Bax effect is dependent on lipid unsaturation index

It is known that ethanolic stress induces alterations of lipids, both in the repartition of phospholipids species and in fatty acid composition [16]. Conversely, lipid composition strongly modifies yeast response to different stresses, including ethanolic stress [17,18].

Sensitivity of lipids to oxidation strongly depends on their unsaturation index. Yeast phospholipids usually contain a low proportion of unsaturated fatty acids but this proportion can be manipulated. For example, the unsaturation index of lipids can be increased by the addition of dioctylphthalate to a homemade minimal medium: the unsaturated/saturated fatty acids ratio is increased from 0.8 to 3.3 [15]. We actually observed that the addition of dioctylphthalate markedly increases kinetics of Bax-induced cell death in a 6% glucose medium (Fig. 5A).

The effect of the addition of dioctylphthalate on Bax-protecting effect on PI permeability of the plasma membrane was then assayed (Fig. 5B). Wild-type strain grown in a minimal medium in the absence of dioctylphthalate (low unsaturation index) did not exhibit any ethanol-induced PI permeability, suggesting that the effect of ethanol was prevented when lipids



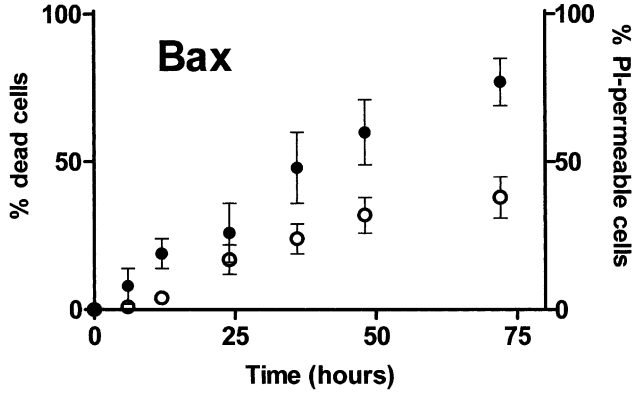
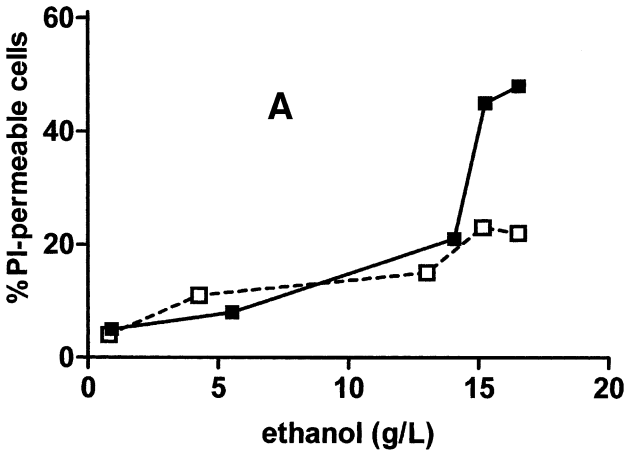


Fig. 3. Correlation between PI permeability and cell death. PI permeability (open symbols) was measured as in Fig. 2. The percentage of dead cells (closed symbols) was determined by plating 200 cells on YPD+doxycycline medium and counting colony formation after 48 h incubation at 28°C.



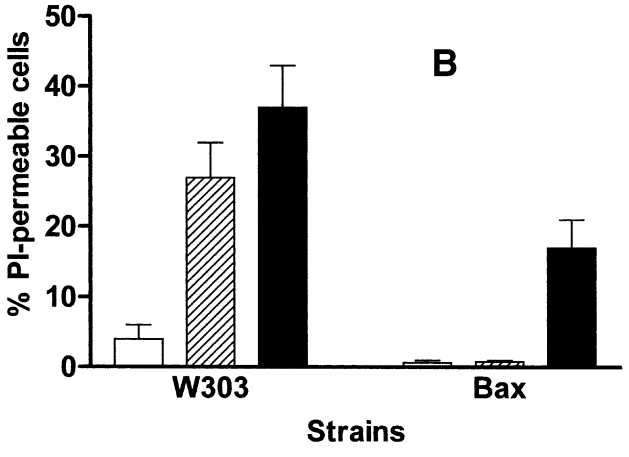


Fig. 4. Ethanol accumulation is responsible for PI permeability. A: Wild-type (■) or Bax-expressing (□) cells were grown for 24 h in a 6% glucose-supplemented medium and PI permeability and ethanol accumulation were measured simultaneously. B: PI permeability of wild-type or Bax-expressing cells was measured under three conditions: 24 h in 0.5% glucose-supplemented medium (open bars), 24 h in 6% glucose-supplemented medium (full bars), 18 h in 0.5% glucose-supplemented medium, addition of 5% ethanol, and a further 6 h growth (dashed bars).

contain mainly saturated fatty acids. As expected, the addition of dioctylphthalate, which restores a high unsaturation index, allowed the same ethanol-induced PI permeability as in semi-synthetic medium (compare Figs. 2 and 5B). Under these conditions, the effect of ethanol was also prevented by Bax expression (Fig. 5B), like in semi-synthetic medium (Fig. 2). From these data, it can be hypothesized that ethanol-induced permeability of the plasma membrane only occurs when fatty acids are mainly unsaturated, and that the Bax effect on plasma membrane permeability is also dependent on fatty acid unsaturation index.

This role of fatty acid unsaturation is in agreement with a possible role of lipid oxidation. This is further supported by the fact that these effects are dependent on the air/medium ratio (Fig. 6, top). The effect of resveratrol, a compound known to inhibit lipid peroxidation, was assayed (Fig. 6, bottom). Resveratrol slightly increased ethanol-induced PI permeability and, most importantly, almost fully prevented the Bax-protecting effect.

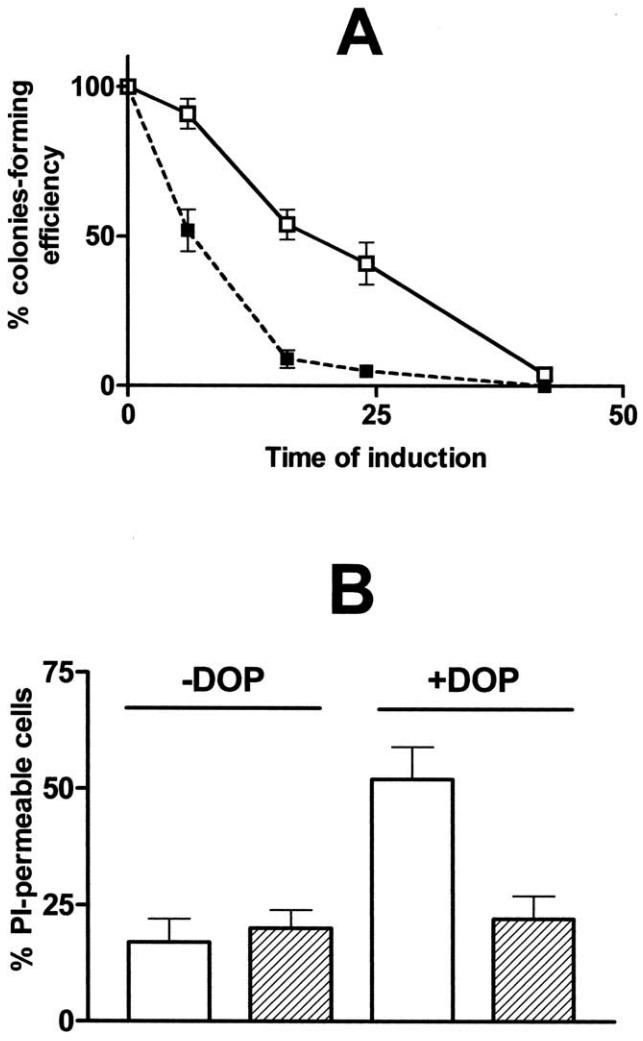


Fig. 5. Effect of dioctylphthalate on Bax-induced cell death and protective effect on PI permeability. A: Bax-expressing cells were pregrown under repressive conditions (+doxycycline). At $t=0$, they were washed and transferred to a homemade minimal medium in the absence of doxycycline, and in the absence (□) or in the presence (■) of 100 μM dioctylphthalate. Cell death was measured as in Fig. 2. B: Wild-type (open bars) or Bax-expressing (full bars) cells were grown for 24 h in the absence or in the presence of 100 μM dioctylphthalate and PI permeability was measured as in Fig. 2.

4. Discussion

The mechanism by which pro-apoptotic members of the Bcl-2 family, such as Bax, are able to kill yeast is yet undefined. Cytochrome *c* relocalization from mitochondria to the cytosol occurs in yeast [12], owing probably to the formation of a channel having the same characteristics as the mitochondrial apoptosis-induced channel [11]. However, cytochrome *c* is not directly involved in yeast death since its absence slows down but does not prevent Bax-induced death [13]. Madeo et al. [5] have reported similarities between Bax-induced cell death and hydrogen peroxide-induced cell death: both induced phosphatidylserine exposure and nuclear DNA fragmentation, but the effects on plasma membrane permeability were not investigated.

Ethanolic stress-induced death normally occurs in fermenting yeast cultures and results especially in alterations of endocytosis [19] and cell wall [20]. Signalling of this stress exhibits functional overlap with other stresses such as heat stress and osmotic stress [14]. The involvement of three heat shock proteins, namely HSP104 [21], HSP30 [22] and HSP12 [23], has been proposed. Metabolic changes induced by these stresses may include trehalose synthesis [24–26], although other authors did not observe a clear correlation between trehalose synthesis and other stress-linked characteristics, such as intracellular acidification [27]. In addition to trehalose synthesis, major changes occur in lipid composition [16,28]. Conversely, lipid composition modulates stress resistance [17,18,26]. It has also been shown that antioxidant defences of the cells are required for the resistance to these stresses [29–31].

Data reported in this paper show that ethanolic stress in wild-type yeast cells is associated to alterations of PI permeability of the plasma membrane, which was in turn clearly correlated to cell death. The expression of the pro-apoptotic protein Bax induces a paradoxical effect: a higher ratio of cell death associated with a decrease of PI permeability. This effect is clearly relevant to the normal function of Bax in mammalian cells, since one major characteristic of apoptosis (versus necrosis) is the maintenance of the permeability bar-

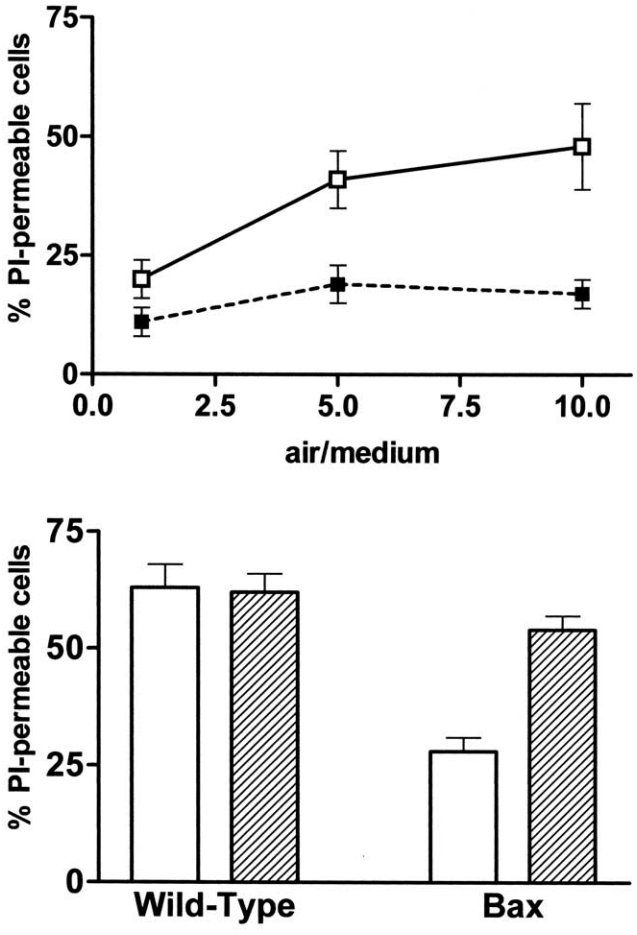


Fig. 6. Protective effect of Bax may depend on lipid oxidation. Top: Wild-type (□) or Bax-expressing (■) cells were grown for 24 h in Erlenmeyer flasks containing different volumes of 6% glucose-supplemented medium. PI permeability was measured as in Fig. 2. Bottom: Wild-type or Bax-expressing cells were grown for 40 h in a 6% glucose-supplemented YNB medium in the absence (empty bars) or presence (dashed bars) of 100 μM resveratrol. PI permeability was measured as in Fig. 2.

rier of the plasma membrane during cell death. Data presented herein strongly support the hypothesis that yeast has the equipment required to engage the cells in an 'apoptosis-like' form of death and that Bax expression is able to activate this equipment. In addition, these data are the first report of a direct effect of Bax on plasma membrane permeability properties.

Ethanol-induced PI permeability is prevented by a low aeration or by a low degree of fatty acid unsaturation: this suggests that this effect is related to lipid oxidation. It should be noted that ethanol-induced cell death of PC12 cells is associated with lipid peroxidation [32], suggesting a conservation of this mechanism from yeast to mammalian cells and showing that yeast can be a powerful model for investigating the molecular mechanisms underlying ethanol effects. Depending both on cell type and on the extent of ethanol treatment, hallmarks of necrosis and apoptosis have been reported in mammalian cells [33,34]. Mitochondrial permeability transition [35] and a role of reactive oxygen species [36] have been reported, which can be involved both in apoptosis and in necrosis.

Interestingly, both the killing efficiency and the effects on plasma membrane permeability of Bax were attenuated in yeast cells containing poorly unsaturated fatty acids. This could be explained either by an interaction of Bax with ethanol stress-induced signalling pathway, or by a more direct effect on lipid metabolism. The fact that the antioxidant resveratrol prevented Bax effects on ethanol-induced plasma membrane permeability supports the latter hypothesis. Interestingly, recent studies reported anti-apoptotic effects of resveratrol [37,38]; however, other studies reported pro-apoptotic properties of this compound [39]. These paradoxical observations might come from multiple targets of resveratrol in different cellular types, as well as the dual function of oxidation products of fatty acids, which can induce cell death via non-selective effects and prevent cell death via selective effects on signal transduction (see [40] for review). The remaining question is to determine which yeast genes are involved in this protecting effect of Bax on plasma membrane permeability. The analysis of yeast mutants resistant to Bax effects will help to answer this question.

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