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Photodynamic treatment of yeast cells with the dye Toluidine blue: all-or-none loss of plasma membrane barrier properties

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Photodynamic treatment of *Kluyveromyces marxianus* with the sensitizer Toluidine blue leads to the loss of colony forming capacity. In this paper, the influence of this treatment on the barrier properties of the plasma membrane has been studied. Photodynamic treatment with the dye Toluidine blue resulted in efflux of potassium ions and E_{260}-absorbing material. Moreover, cells became stainable with erythrosine. It is concluded that the permeability change induced by photodynamic treatment proceeds in an all-or-none fashion. Treatment of this yeast strain, with the dye and light, also induced a diminution of the cell volume. This process is most likely not coupled to the cellular potassium content, but rather to the integrity of the vacuole. These data suggest that the vacuole has an important function in the maintenance of cell volume. Finally, it was observed that the loss of cell viability was not induced by the all-or-none loss of barrier properties.

Introduction

Toluidine blue is a positively charged dye that under physiological conditions does not, or very slowly, enter the yeast cell [1]. It is, however, adsorbed to negatively charged groups on the cell surface. Previous studies have indicated that TB binds specifically to periplasmatically localized polyphosphates [2].

Irradiation of TB in the presence of oxygen leads to the generation of reactive oxygen species of which singlet oxygen seems to be the predominant one [1]. Due to this property of TB, photodynamic treatment of yeast with this sensitizer causes loss of colony formation [1]. The mechanism of this process is not fully understood. It seems unlikely that genetic alteration is the primary cause of loss of clonogenicity [1]. Based on its localization it has been suggested that the plasma membrane is the primary target of TB-induced photodynamic action [1]. Support for the hypothesis that the plasma membrane is the main target of photodynamic treatment is scarce.

It is the aim of this paper to determine whether photodynamic treatment with TB causes membrane damage. It will be shown that drastic permeability changes occur as a consequence of photodynamic treatment and that all-or-none effects are involved. Experimental evidence indicates, however, that this loss of barrier properties is not causally related to loss of clonogenicity.

Materials and Methods

Yeast cells of the diploid strain *Kluyveromyces marxianus* CBS 397 were grown aerobically in a complex medium containing: 1% bacto-peptone, 1% yeast extract and 2% glucose. After 19 h of incubation at 30°C (early stationary phase), cells were harvested and washed two times with deionized water. For photodynamic treatment the cells were resuspended in 1 mM Tris-HCl (pH 7.2) at a cell density of 0.5% wet wt./vol. A 10-ml aliquot of the cell suspension was transferred to a 25 ml Erlenmeyer flask and Toluidine blue O (Serva) was added to a final concentration of 1 μg/ml. After 20 min of incubation in the dark the cells were exposed to light. The illumination was performed with a 150 W halogen lamp. The light was filtered with a Kodak wratten No. 8 filter cutting off at wavelengths < 500 nm. The fluency was about 300 W/m² as measured by a photometer (EG&G Electro-optics model

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Abbreviations: CTAB, cetyltrimethylammonium bromide; TB, Toluidine blue.

450-1). During illumination the suspension was stirred magnetically.

Potassium in the medium was determined with a Corning clinical flamephotometer after centrifugation of the cell suspension. The amount of potassium liberated by adding CTAB (0.2 mg/ml final concentration) to the suspension was taken as 100%.

The percentage of erythrosine-stainable cells was determined in samples diluted in water and stained with 0.25% erythrosine. Cell counting was performed immediately after staining.

The cell volume was determined with a Coulter Multisizer II equipped with a 30 μm orifice. Directly after taking a sample and diluting it in 0.45% NaCl cell size was measured.

Arginine release from the cells was measured, using [^{14}C]arginine-labelled yeast. To this purpose, a 1% cell suspension in 50 mM Tris-maleate buffer (pH 4.9) containing 0.5% ethanol was labelled with [^{14}C]arginine (Amersham) at 30°C. After 25 min of incubation the suspension was centrifuged, whereafter the pellet was washed with deionized water, centrifuged and the cells were resuspended in 1 mM Tris-HCl (pH 7.2) at a cell density of 0.5% wet wt./vol. Photodynamic treatment was performed as described in the previous section. Samples were taken, centrifuged and 0.4 ml supernatant was used for liquid scintillation counting. The amount of arginine released by adding CTAB (0.2 mg/ml final concentration) to the suspension was used as 100%. With this method about 65% of accumulated arginine could be released.

Cell survival was determined by taking 10 μl samples at appropriate times before and during illumination, diluting these samples to 5000 cells/ml and spreading 500 cells at solidified (1% agar) medium. The number of colonies was counted after two days incubation in the dark at 30°C.

Cytophotometry was performed essentially according to Van der Ploeg et al. [3]. To prevent refraction, air dried specimen were embedded in Fluoromount (UDH) (refractive index 1.49). Stepping size was 0.5 μm and a bandpass filter of 610 nm was used.

Reproducibility of the results. All the experiments were performed at least three times, and the data reported here are average values.

Results

Cell survival and leakage of intracellular compounds

The effect of photodynamic treatment on the survival of yeast cells was measured and compared with K^+ loss. Fig. 1 shows that photodynamic treatment with TB causes a rapid decrease of cell survival. After 10 min of illumination more than 98% of the cells had lost the ability to form colonies. This treatment also results in the loss of potassium from the cells. K^+

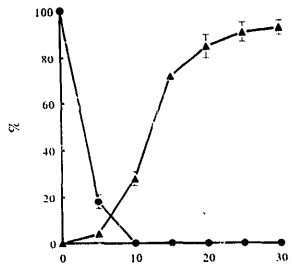

Fig. 1. Cell survival and potassium loss during photodynamic treatment. Colony formation (●) and K^+ loss (▲).

leakage started after a lag time of 5 min and was complete after about 30 min.

During this illumination period the cells did not only loose potassium but also other compounds, like nucleotides and other substances absorbing at 260 nm. Fig. 2 shows that during light exposure the percentage of potassium loss parallels that of 260 nm absorbing material. This indicates that the permeability change induced by photodynamic treatment is not specific, but rather seems to have a more general character.

Cessation of light exposure under conditions where only a part of the potassium and 260 nm absorbing

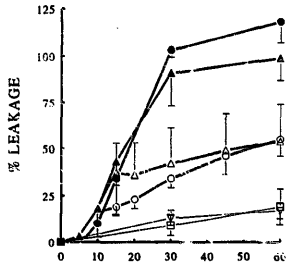

Fig. 2. Potassium leakage and release of E_{260}-absorbing material during photodynamic treatment. Loss of K^+ (▲) and E_{260}-absorbing material (●) during illumination in the presence of TB. Loss of K^+ (△) and E_{260}-absorbing material (○) during illumination in the presence of TB, light switched off after 15 min. Loss of K^+ (□) and E_{260}-absorbing material (▽) in the presence of TB without illumination.

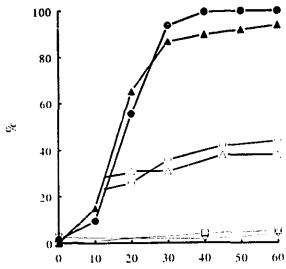

Fig. 3. Cell staining and potassium leakage during photodynamic treatment. Cells stainable with erythrosine (●) and K^+ loss (▲) during illumination in the presence of TB. Stainable cells (○) and K^+ loss (△) during illumination in the presence of TB, light switched off after 12 min. Cells stainable with erythrosine (▽) and K^+ loss (□) in the presence of TB without illumination.

material had leaked out resulted in a decreased rate of loss of these compounds, compared to prolonged illumination (Fig. 2). This shows that continuous light exposure is necessary to achieve maximal release of K^+ and E_{260} absorbing material.

Erythrosine staining

To further characterize the change in membrane permeability, the effect of photodynamic treatment on stainability of the yeast with erythrosine was measured. Erythrosine is a dye, not taken up by the intact cell [4]. Only when a drastic permeability change of the plasma membrane occurs, this dye can enter the cell. Fig. 3 shows that in the course of photodynamic treatment the percentage of erythrosine-stainable cells was about equal to the percentage potassium lost from the cells, both with continued and with interrupted light exposure. This strongly suggests that photodynamic treatment caused a non-specific perturbation of the barrier properties of the cell membrane.

Cell volume

Potassium loss from yeast, caused by various agents, often appears to be accompanied by a decrease in cell size, suggesting a correlation between K^+ content and cell volume [5]. Fig. 4A shows that photodynamic treatment with TB also caused a decrease in cell volume. However, after 20 min of illumination, 70% of potassium had leaked out of the cell whereas no change in cell volume was observed. Only a prolonged light exposure induced a volume decrease. These data suggest that under our conditions a strict correlation between cellular K^+ levels and cell volume did not occur. This conclusion was corroborated by the experiment shown in Fig. 4B, where the light was switched off after 20 min of illumination. A nearly complete potassium loss was observed with no decrease of cell volume. Only after resuming light exposure cell shrinkage took place.

Arginine release

Arginine is taken up by yeast cells and is accumulated mainly in the vacuole [6]. The yeast cell contains

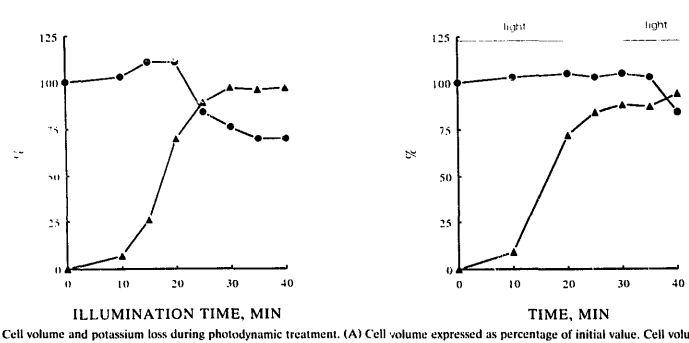

Fig. 4. Cell volume and potassium loss during photodynamic treatment. (A) Cell volume expressed as percentage of initial value. Cell volume (●) and K^+ leakage (▲). (B) Cell volume (●) and potassium leakage (▲) during light exposure in the presence of TB, illumination interrupted after 20 min and continued at time 30 min.

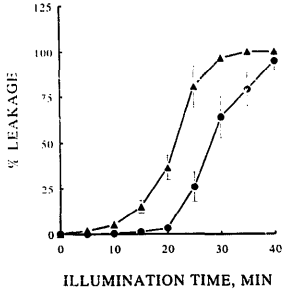

Fig. 5. Arginine release and potassium loss during photodynamic treatment. Arginine release (●) and K^+ loss (▲).

two arginine pools: a pool of free arginine accumulated in the vacuole and a non-releasable pool formed by arginine build into proteins. Due to the accumulation of free arginine in the vacuole, arginine leakage from yeast can be used as a measure of damage of the vacuolar membrane. Arginine-prelabelled cells were illuminated and the release of potassium and [^{14}C]arginine was determined. It was found that the release of arginine did not equal the loss of potassium (Fig. 5). Arginine loss started after 20 min of light exposure and reached a maximum level after 40 min. This indicates that arginine is retained in the vacuole during the first 20 min of illumination, and that the vacuolar membrane looses its barrier property at a later point of time than the plasma membrane.

Cytophotometry

Scanning microphotometric measurements can be used to determine the cellular localization of TB, as was shown before by Ito [1] for *Saccharomyces cerevisiae*. We obtained identical results for *K. marxianus* cells and TB at a concentration of 10 μg/ml. In cells kept in the dark no absorption above the background could be detected whereas cells exposed to 30 min of light showed a faint absorption. The latter suggests intracellular localization of the dye. However, with the TB concentration as used in all other experiments (1 μg/ml), no absorption could be measured.

Discussion

Leakage of intracellular contents induced by a cell damaging agent indicates deterioration of the barrier function of the membrane. In general a partial leakage of intracellular constituents can be caused either by a graded response of all cells, or, alternatively, by an all-or-none response of a limited number of cells. In the latter case the population will consist of a mixture of completely leaky and completely intact cells.

In yeast many compounds give rise to efflux of potassium. This applies to detergents [7–9], organic dyes [10,11], inhibitors of plasma membrane ATPase [12–14], polycations [15] and heavy metal ions [16,17]. Many of these compounds provoke an all-or-none response [7–10,16], while other compounds induce a combined all-or-none response and a graded release of potassium [5,17,18].

K^+ loss from yeast cells induced by TB and light has, to our knowledge, not been described yet. The results presented in this paper show that photodynamic treatment with TB causes release of potassium and E_{260} absorbing material in an all-or-none fashion. First, cessation of illumination resulted in a decrease of further leakage, to a rate comparable to the control (Fig. 2). This can easily be reconciled with an all-or-none mechanism, but not with a graded response. In the latter case the already inflicted membrane damage in all cells would be expected to cause an ongoing K^+ leakage in the absence of light. Second, the fact that photodynamic treatment causes a parallel change in permeability for potassium, E_{260}-absorbing material and erythrosine, both in continuous and in interrupted illumination, is difficult to reconcile with a graded process. The simultaneous change of permeability for these compounds, differing in size and charge, can easily be explained by an all-or-none mechanism. The conclusion is that photodynamic treatment with TB causes an all-or-none disruption of the yeast plasma membrane barrier.

Potassium loss from yeast can be accompanied by cell shrinkage. Addition of ATPase inhibitors results in K^+ efflux and a decrease in cell volume [5]. The results in Fig. 4A show that in the case of photodynamic treatment potassium loss and cell volume change are not correlated. Fig. 4B is even more convincing: when illumination was interrupted after 20 min, no cell shrinkage took place during post-incubation in the dark. Only after resuming light exposure a decrease in cellular volume was measured. Therefore, it appears likely that under our conditions the potassium content is not the main cell size determining factor. The fact that cell shrinkage needs longer illumination times than the disruption of the plasma membrane barrier suggests that cell size is determined by cellular sites that are less susceptible to, and/or accessible for TB generated singlet oxygen. It is tempting to suggest that these sites are located intracellularly, which is in agreement with the observation that TB only enters the cell after prolonged illumination [1]. Therefore, as a possible regulator of volume, cell organelles, like the vacuole, can be postulated. Experimental support for this hypothesis comes from the arginine release experi-

ments, showing that the vacuolar arginine pool is released after the major plasma membrane disruption. Comparison of Figs. 4A and 5 shows, moreover, a close parallel between arginine leakage and cell shrinkage. These data suggest that the vacuole is involved in determining the size of the yeast cell. The delayed leakage of arginine must be expected because of the initial peripheral location of TB. After major damage of the plasma membrane the dye will enter the cells with subsequent photodynamic damage of the vacuolar membrane.

It has been suggested that plasma membrane damage is responsible for loss of clonogenicity induced by TB and light [1,19,20]. This paper shows that a drastic change of plasma membrane permeability occurs in an all-or-none fashion but that such a loss of barrier properties is probably not responsible for the reduced viability of the yeast (Fig. 1). Still, considering the peripheral location of TB it seems obvious that the plasma membrane must be the primary target of photodynamically-induced loss of clonogenicity, as the short lifetime of the generated singlet oxygen will not allow substantial damage of intracellular targets [21]. Therefore, not the loss of the barrier function, but other, more subtle forms of membrane damage will probably be involved in photodynamically-induced loss of clonogenicity. It is possible that a subtle modification of the plasma membrane changes its permeability characteristics in such a way that a small fraction of the dye enters the cell. This was suggested by Bertoloni et al. [22] for treatment of *Candida* with haematoporphyrin. At a concentration of 10 μg/ml TB we indeed observed a reallocation after illuminating for 30 min. Since at a concentration of 1 μg/ml no absorption could be measured, no conclusion can be drawn on the possible reallocation of Toluidine blue. Further studies will have to reveal the exact mechanism of photodynamic inactivation of *K. marxianus* cells.

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