

Aspirin commits yeast cells to apoptosis depending on carbon source

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The effect of aspirin on the growth of a wild-type *Saccharomyces cerevisiae* strain (EG103), containing both copper,zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD), a strain deficient in MnSOD (EG110) and a strain deficient in CuZnSOD (EG118) was measured in media containing different carbon sources. Aspirin inhibited the fermentative growth of all three strains in glucose medium. It inhibited the non-fermentative growth of the MnSOD-deficient strain very drastically in ethanol medium and had no effect on this strain in glycerol or acetate medium. The non-fermentative growth of the other two strains was not affected by aspirin. The growth inhibition of strain EG110 was associated with early necrosis in glucose medium and late apoptosis in ethanol medium. The apoptosis was preceded by a pronounced loss of cell viability. The growth inhibitory effect of aspirin was not reversed by the antioxidants *N*-acetylcysteine and vitamin E. Furthermore, aspirin itself appeared to act as an antioxidant until the onset of overt apoptosis, when a moderate increase in the intracellular oxidation level occurred. This suggested that reactive oxygen species probably do not play a primary role in the apoptosis of cells exposed to aspirin.

Received 18 June 2003

Revised 18 September 2003

Accepted 30 September 2003

INTRODUCTION

Non-steroidal anti-inflammatory drugs such as aspirin can prevent or inhibit the development of colorectal cancer (for review, see Shiff & Rigas, 1997). These drugs have been shown to induce apoptosis in colon cancer cell lines (Elder *et al.*, 1996; Castano *et al.*, 1999; Goel *et al.*, 2003). We studied the effect of aspirin on cell growth and its propensity to induce apoptosis in a model system provided by yeast cells. In *Saccharomyces cerevisiae*, apoptosis is characterized by the hallmarks of mammalian apoptosis, including externalization of phosphatidylserine at the surface of the cytoplasmic membrane, membrane blebbing, chromatin condensation and margination and DNA cleavage (Madeo *et al.*, 1997). Recently, a yeast protein with structural homology to mammalian caspases (YOR197Wp) was found to function as a caspase in yeast (Madeo *et al.*, 2002).

The apoptotic phenotype observed in the cell-cycle mutant *cdc48*^{S565G} of *S. cerevisiae* is associated with accumulation of reactive oxygen species (ROS) (Madeo *et al.*, 1999). ROS also accumulate in yeast cells undergoing apoptosis induced by mammalian Bax (Ligr *et al.*, 1998). Furthermore,

apoptosis is induced by growth of a *gsh1* deletion mutant in the absence of glutathione and by exposure of yeast cells to low concentrations of H₂O₂. These findings have led to the conclusion that production of ROS is a necessary and sufficient condition for the induction of apoptosis in yeast (Madeo *et al.*, 1999).

Aspirin is known to scavenge ROS (Saini *et al.*, 1998), yet it induces apoptosis in cancer cell lines (Elder *et al.*, 1996; Castano *et al.*, 1999; Goel *et al.*, 2003). In the present work, we studied the effect of aspirin on yeast cells with differential protection against ROS, as obtained with wild-type, cytoplasmic copper,zinc superoxide dismutase (CuZnSOD, SOD1)-deficient and mitochondrial manganese superoxide dismutase (MnSOD, SOD2)-deficient strains, and with differential production of ROS as obtained with growth on fermentable and non-fermentable carbon sources. In general, we found that aspirin induced apoptosis in MnSOD-deficient cells growing on ethanol as the non-fermentable carbon source. Our results suggest that generation of ROS may be a relatively late event and not the initial cause of the observed cell death process.

METHODS

Cell cultures and treatments. The *S. cerevisiae* strains used in this study were EG103 (*MATα leu2-3 112 his3Δ1 trp1-289a ura3-52 GAL⁺*), EG110 (EG103 *sod2Δ::TRP1*) and EG118 (EG103

Abbreviations: CuZnSOD, copper,zinc superoxide dismutase; DCDHF-DA, 2',7'-dichlorodihydrofluorescein diacetate; FACS, fluorescence-activated cell sorting; MnSOD, manganese superoxide dismutase; PI, propidium iodide; ROS, reactive oxygen species.

sod1Δa::URA3), kindly provided by Edith Gralla of UCLA and Valeria C. Culotta of Johns Hopkins University. Cells were grown in enriched yeast extract, peptone-based medium with 2% (w/v) glucose (YEPD), or 3% (v/v) ethanol (YPE), or 3% (v/v) glycerol (YEPG) or 2% (w/v) potassium acetate (YPA). For plates, 2% agar was used and incubation was at 28 °C. Aerobic growth in liquid culture was maintained at 28 °C with constant shaking at 300 r.p.m. The cells were also cultured in fresh media in the presence of 15 mM aspirin (acetylsalicylic acid) (Sigma). In this case, with the exception of YPA where the pH was already 5.8, the pH of the media was adjusted to 5.5 by using 1 M Trizma base (Sigma). For antioxidant treatment, 4 mM *N*-acetylcysteine (Sigma) and 25 μM vitamin E (α -tocopherol acid succinate) (Sigma) were added to the cell cultures.

Measurement of cell growth and cell viability. Cell growth was measured as the optical density at 600 nm. Cultures with OD₆₀₀ values greater than 1.0 were diluted as necessary. The dilutions were in the range 1 in 10 to 1 in 20. Since a viable cell is defined as one that can form a colony on rich YEPD medium, cell viability was measured by plating serial dilutions of the treated and untreated yeast cultures onto YEPD plates to find the number of cells (~300) to be plated, as determined by counting colonies (Longo *et al.*, 1996). The percentage of colony-forming units (c.f.u.) of cells treated with aspirin was obtained by relating the c.f.u. counts of treated cells to those of untreated cells, which were considered to be 100%.

Detection of apoptosis or necrosis. Quantification of apoptosis by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis, using a Bio-Rad BRYTE HS flowcytometer, was performed as described previously (Sazer & Sherwood, 1990). Annexin-V-Fluorescein (Annexin-V-Fluos Staining Kit; Roche Diagnostics) and PI staining were essentially performed as described previously (Madeo *et al.*, 1997), with some modifications, and FACS analysis was carried out. Yeast cells (1×10^7) were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl₂, 35 mM potassium phosphate, pH 6.8), incubated with Tris/DTT buffer (100 mM Tris/Cl, pH 9.4, 10 mM DTT) for 15 min at 30 °C with gentle shaking to break cysteine bridges, washed again in sorbitol buffer, digested with Zymolase-20T (Seikagru Corporation) [5 mg (g wet weight of cells)⁻¹] in sorbitol buffer for 2 h at 30 °C, harvested, washed in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) containing 1.2 M sorbitol, harvested and resuspended in 100 μl of binding buffer/sorbitol. Annexin-V-Fluos (10 μl) and 10 μl PI (500 μg ml⁻¹) were added and the cells were incubated for 20 min at room temperature. The cells were harvested, suspended in 0.5 ml of binding buffer and analysed on the Bio-Rad BRYTE HS flowcytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter >560 nm for PI detection.

Measurement of intracellular oxidation level. Intracellular ROS were detected by using the oxidant-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCDHF-DA) (Molecular Probes). One millilitre of yeast cell culture (10^7 cells), to which was added 2 μl of DCDHF-DA from a fresh 5 mM stock solution in ethanol, was incubated at 28 °C for 20 min. The cells were then washed twice in sterile distilled water and resuspended in 1 ml of 50 mM Tris/Cl buffer, pH 7.5. Two drops of chloroform and 1 drop of 0.1% (w/v) SDS were added and the cells were vortexed for 20 s and allowed to stand for 15 min to allow the dye to diffuse into the buffer. The cells were pelleted and the fluorescence of the supernatant was measured using a Bio-Tek Instruments fluorometer with excitation at 490 nm and emission at 518 nm.

RESULTS

Effect of aspirin on the growth and viability of *S. cerevisiae* cells is affected by the carbon source in the medium

Growth curves for the *S. cerevisiae* strains EG103 (containing both MnSOD and CuZnSOD), EG110 (deficient in MnSOD) and EG118 (deficient in CuZnSOD) cultivated on fermentable and non-fermentable media, in the absence or presence of 15 mM aspirin, are presented in Fig. 1. In medium with glucose as the fermentable carbon source (YEPD), aspirin inhibited the growth of the three strains (Fig. 1a–c). In contrast, in medium with ethanol as the non-fermentable carbon source (YPE), aspirin drastically inhibited the growth of EG110 cells (Fig. 1e), yet it had no significant effect on EG103 (Fig. 1d) and EG118 cells (Fig. 1f). In medium containing glycerol (YEPG), aspirin had no effect on the growth of any of the strains (Fig. 1g–i), including EG110 (Fig. 1h), even though glycerol is also a non-fermentable carbon source. Similarly, in acetate medium (YPA), aspirin had no effect on the growth of the three strains (Fig. 1j–l). Thus, aspirin had a carbon-source-dependent and MnSOD-dependent effect on yeast cell growth.

To determine whether the decrease in optical density of aspirin-treated cells reflected growth inhibition or loss in cell viability and possibly cellular death, we measured cell viability by plating serial dilutions of the cultures onto YEPD plates and counting the resulting colonies. Fig. 2(a, b) shows that there was a drastic drop in EG110 cell viability (c.f.u. %) after 48 h growth in YPE medium and after 22 h growth in YEPD medium. Thus, the decreased growth of aspirin-treated EG110 cells can be attributed primarily to a decrease in cell viability.

Aspirin-treated MnSOD-deficient *S. cerevisiae* cells undergo apoptosis in YPE medium and necrosis in YEPD medium

Next, we examined whether the death of EG110 cells was due to apoptosis or necrosis by FACS analysis of PI-stained cells (Fig. 3a) grown in YPE medium in the absence (upper panels) or presence (lower panels) of 15 mM aspirin. Cells with less PI staining than that of G₁ cells are considered apoptotic. In EG110 cells growing in YPE medium in the presence of 15 mM aspirin, more than 50% of the cells were apoptotic after a cultivation time of 168 h, whereas only ~8% of the untreated cells were apoptotic.

These results were confirmed by studying the externalization of phosphatidylserine with the Annexin-V-binding assay (van Engeland *et al.*, 1998). Apoptotic and necrotic cells were distinguished by double-labelling for Annexin-V-Fluos (green dye) and PI (red dye), which is a membrane-impermeable DNA stain. Fig. 3(b) shows that after 168 h cultivation in YPE medium, more than 50% of EG110 cells treated with aspirin showed high Annexin and low PI

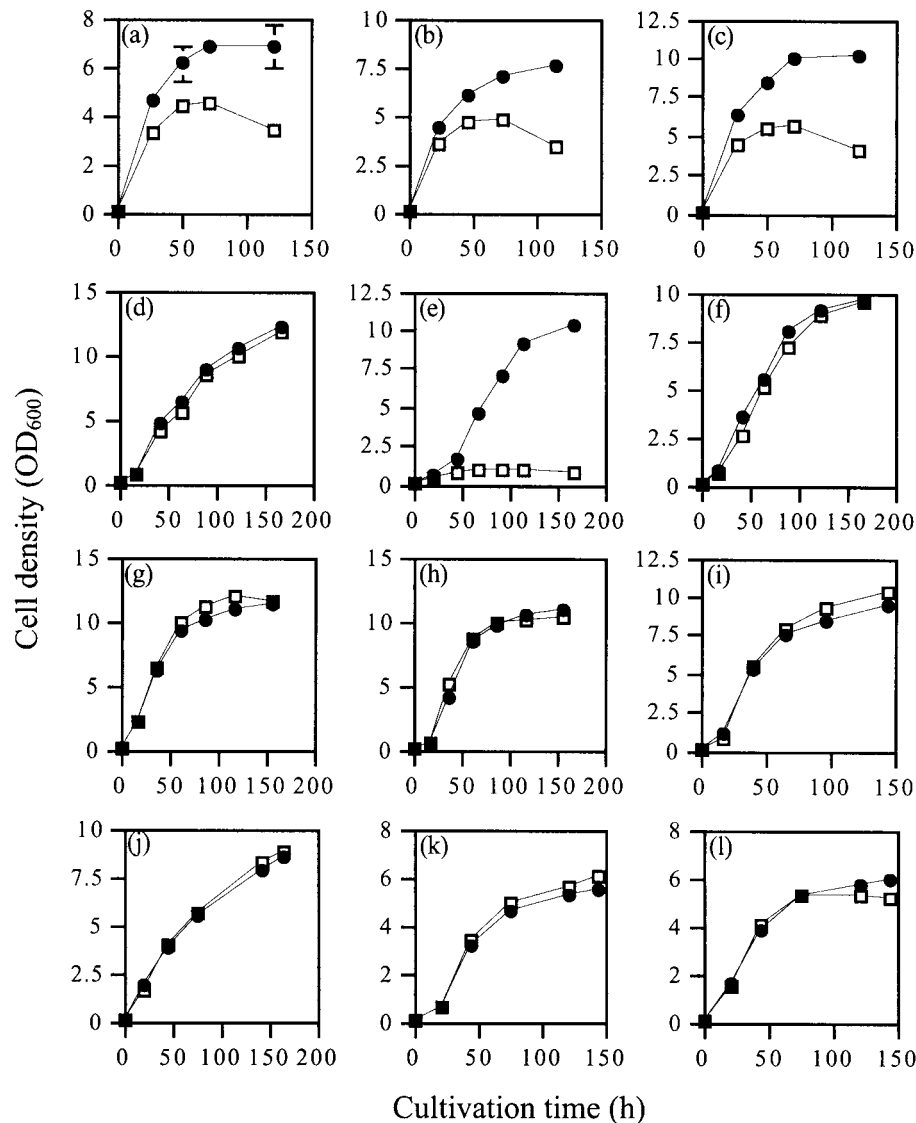


Fig. 1. Exponential growth curves for *S. cerevisiae* strains EG103 (containing both MnSOD and CuZnSOD), EG110 (deficient in MnSOD) and EG118 (deficient in CuZnSOD), shown respectively from left to right, in YEPD (a–c), YPE (d–f), YEPG (g–i) and YPA (j–l) medium. The yeast cells were grown in the absence (●) and presence (□) of 15 mM aspirin. Each point represents the mean of at least four independent determinations. Error bars are ± 1 SD and appear where sufficiently large.

staining, and hence were apoptotic (right panel), whereas in the untreated cells only 10 % of the cells were apoptotic (left panel). Thus, in the MnSOD-deficient yeast cells, after 168 h cultivation in YPE medium in the presence of aspirin, the percentage of apoptotic cells increased fivefold compared to the untreated cells.

The mode of cell death was also investigated in EG110 cells growing in YEPD medium in the presence of 15 mM aspirin. After 24 h, 94 % of these cells had high Annexin and high PI staining, and thus were necrotic (Fig. 4b, right panel). The percentage of aspirin-treated cells showing apoptosis was very low, less than 2 % (Fig. 4a, b, right

panels). As expected, untreated cells showed no characteristics of apoptosis or necrosis (Fig. 4a, b, left panels). Thus, in the presence of aspirin, MnSOD-deficient cells appear to die through an early necrotic process in the presence of glucose and through a late apoptotic process in the presence of ethanol.

Aspirin exerts an antioxidant effect but still commits MnSOD-deficient yeast cells to apoptosis in YPE medium

We asked if aspirin could be acting as a pro-oxidant committing the MnSOD-deficient EG110 cells growing in

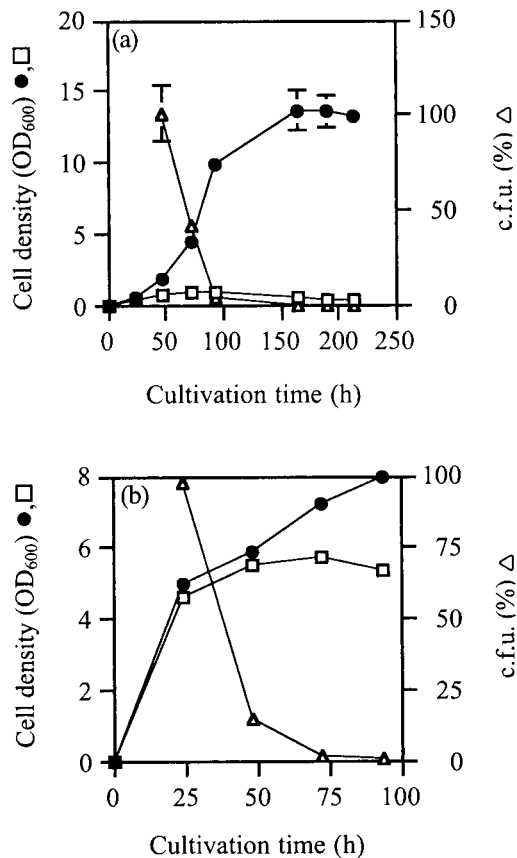


Fig. 2. Exponential growth curves for *S. cerevisiae* EG110 cells (deficient in MnSOD) growing in (a) YPE medium and (b) YEPD medium in the absence (●) and presence (□) of 15 mM aspirin, together with viability measured as percentage of colony-forming units (c.f.u.) of aspirin-treated relative to untreated cells on YEPD plates (Δ). Viability was monitored at the indicated hours by plating ~300 cells from each culture onto YEPD plates and counting the colonies formed. Each point represents the mean of at least four independent determinations. Error bars are ± 1 SD and appear where sufficiently large.

YPE medium to apoptosis, by monitoring growth of these cells in the presence of aspirin along with the antioxidants *N*-acetylcysteine and vitamin E. The antioxidants did not reverse the deleterious effects of aspirin (data not shown). Indeed, studies of the intracellular oxidation levels of EG110 cells by the oxidant-sensitive probe DCDHF-DA revealed a significant antioxidant effect of aspirin on the yeast cells growing in YPE medium up to a cultivation time of 93 h (Fig. 5). However, at 165 h of cultivation time, a moderately significant increase in the percentage relative fluorescence of cells treated with aspirin was observed. This corresponded with the high level of apoptotic cells observed at this stage in the FACS analysis using PI staining (Fig. 3a, lower panels, extreme right). The antioxidant effect of aspirin in EG110 cells was not restricted to growth on YPE medium; it was also found in EG110 cells growing in YEPG

medium (data not shown), in which case aspirin had no overall effect on cell growth (Fig. 1g–i). Thus, aspirin acts as an antioxidant in MnSOD-deficient yeast cells, irrespective of its effect on growth, in YPE or YEPG medium.

DISCUSSION

Our results showed that the effect of aspirin on the growth of wild-type and CuZnSOD- or MnSOD-deficient strains of *S. cerevisiae* depends on the carbon source in the medium. Aspirin inhibited fermentative growth in YEPD medium independently of the expression of CuZnSOD or MnSOD (Fig. 1a–c). This growth inhibition can be attributed to inhibition of glucose transport and utilization in YEPD medium, based on the work of Scharff *et al.* (1981, 1982) which demonstrated that the aspirin metabolite salicylic acid inhibited ATP generation by glycolysis. The MnSOD-deficient strain was of special interest in our studies because of the nature of the growth effects of aspirin in non-fermentable media. Strain EG110 showed early necrosis when grown fermentatively on glucose (Fig. 4b). In fact, necrosis is generally expected as the mode of death in cells deprived of ATP (Leist *et al.*, 1997).

In non-fermentable ethanol medium, MnSOD-deficient cells that were not treated with aspirin showed a small amount of late apoptosis (Fig. 3a). This was not unexpected because mitochondrial electron transport in non-fermentatively growing cells is a major source of ROS production (Longo *et al.*, 1996). Moreover, MnSOD-deficient cells are not protected against ROS generated in the mitochondria. In fact, van Loon *et al.* (1986) found a yeast mutant lacking MnSOD to be hypersensitive to 100% oxygen. Ethanol is expected to increase the mitochondrial production of ROS in yeast cells (Costa *et al.*, 1997). However, it would appear that the condition of MnSOD deficiency, combined with non-fermentative growth on ethanol, did not induce significant apoptosis unless the cells also were treated with aspirin (Fig. 3). The possibility that aspirin caused increased generation of ROS was excluded by the lack of effect of the antioxidants *N*-acetylcysteine and vitamin E on growth or apoptosis. Furthermore, aspirin was found to act as an antioxidant until the appearance of apoptosis, when there was a moderately significant increase in the intracellular oxidation level (Fig. 5). Generation of ROS as a relatively late event, with the ROS not acting as effectors of cell death but possibly as late signals of the apoptotic process, has been observed in neural cells (Schulz *et al.*, 1996). In myeloid leukaemia cells, Cai & Jones (1998) observed that increased cellular oxidation levels occurred in parallel with caspase activation after mitochondrial release of cytochrome *c*. These findings can be extended, with caution, to yeast cells. The involvement of mitochondria in yeast cell apoptosis is evidenced by a decrease in the membrane potential, dysfunction of the mitochondrial proton pump, and release of cytochrome *c* to the cytoplasm in cell death mediated by deletion of the histone chaperone *ASF1/CIA1*

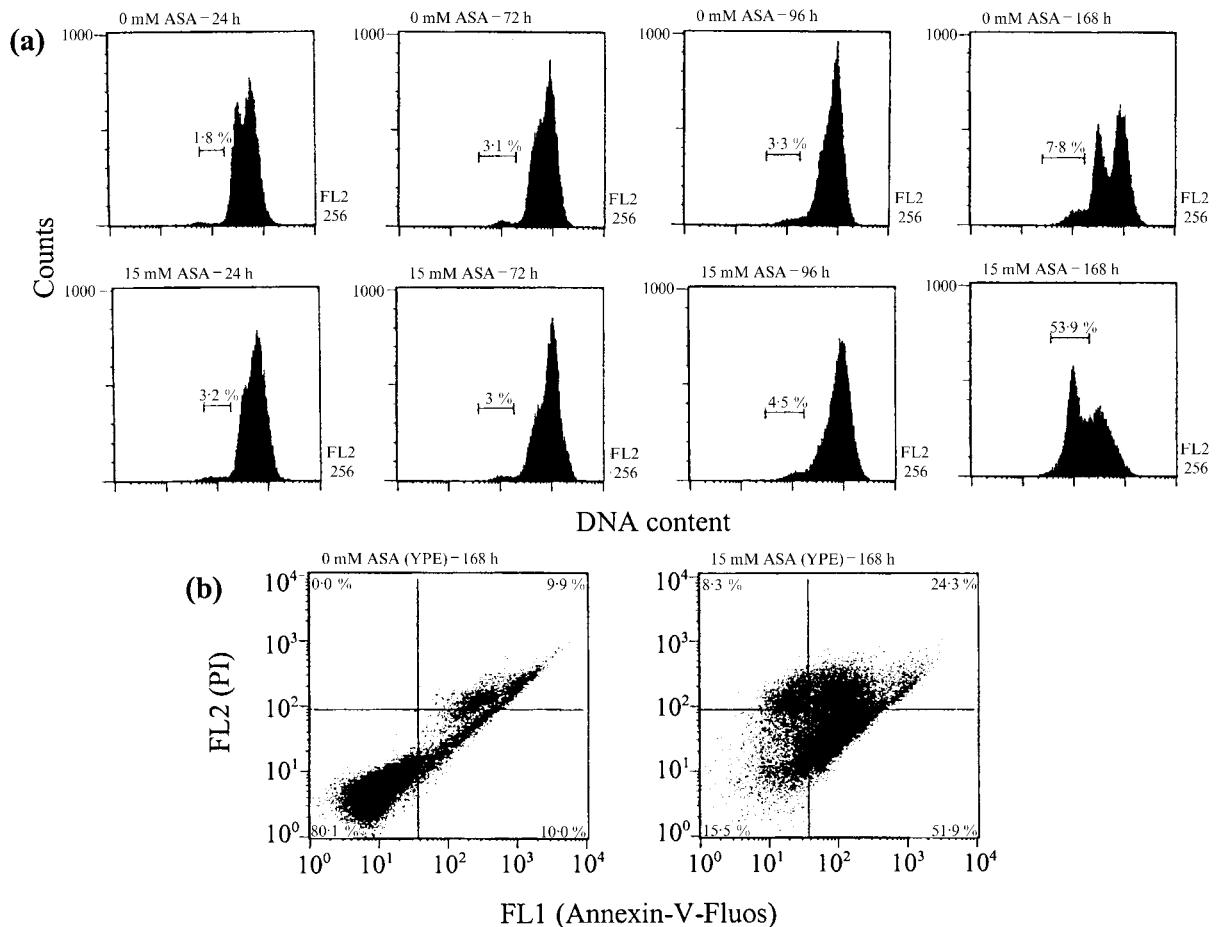


Fig. 3. Induction of apoptosis by aspirin in *S. cerevisiae* EG110 cells (deficient in MnSOD) growing in YPE medium. Analysis of apoptosis was carried out by (a) PI staining and (b) double-labelling for Annexin-V-Fluos and PI. In both types of experiment, FACS analysis was used. The upper and lower panels in (a) show the quantification of DNA content in control cells and in cells treated with 15 mM aspirin, respectively. Data are represented as cell number (Counts) versus fluorescence intensity (DNA content). The per cent value corresponds to the percentage of apoptotic cells. (b) Shows representative dot plots of Annexin-V-Fluos versus PI fluorescence for control cells (left panel) and cells treated with 15 mM aspirin (right panel). Quadrants: lower left, viable cells; lower right, apoptotic cells; upper right, necrotic cells. Approximately 25 000 cells were analysed for each sample. ASA denotes aspirin.

(Yamaki *et al.*, 2001). These findings have been confirmed in stationary phase yeast cells that are committed to apoptosis by acetic acid (Ludovico *et al.*, 2002).

The observed antioxidant effect of aspirin does not support a primary role for ROS in the apoptosis of MnSOD-deficient cells growing on ethanol medium. A drastic fall in the viability of these cells, as measured by the ability to form new colonies (Fig. 2a), occurred much earlier than apoptosis when the antioxidant effect of aspirin (Fig. 5) was at its highest. It would seem that most of the cells were irrevocably committed to death before the actual characteristics typical of apoptosis, such as DNA cleavage, had set in.

Our finding that the presence of MnSOD in *S. cerevisiae* cells, such as EG103 and EG118, protected the cells from

growth inhibition suggests a mitochondrial target for aspirin. Cytoplasmic CuZnSOD is expected to compensate to some extent for the absence of mitochondrial MnSOD in cellular protection against ROS (Longo *et al.*, 1996). However, the presence of CuZnSOD in EG110 cells did not protect the cells from commitment to apoptosis. This throws further doubt on a primary role of ROS in the process. Because of growth inhibition culminating in apoptosis, the question arises as to whether MnSOD-deficient cells were less able to maintain the intracellular level of NADPH needed for reductive biosynthesis, apart from energy requirements, when grown on ethanol than when grown on glycerol or acetate, which appeared to provide full protection against growth inhibition by aspirin. We propose to address this question experimentally in future work.

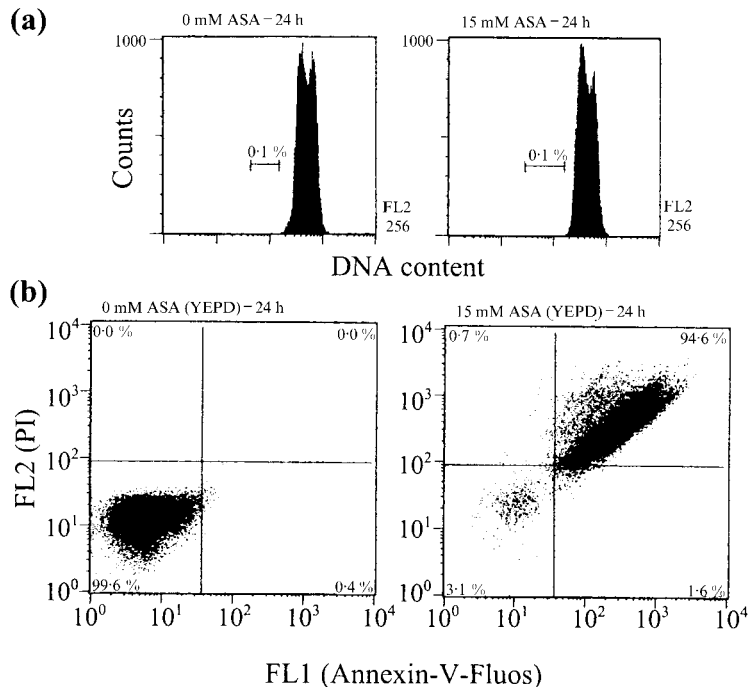


Fig. 4. Induction of necrosis by aspirin in *S. cerevisiae* EG110 cells (deficient in MnSOD) growing in YEPD medium. Analysis of apoptosis was carried out by (a) PI staining and (b) double-labelling for Annexin-V-Fluos and PI. Analysis of necrosis was carried out by double-labelling for Annexin-V-Fluos and PI. In both types of experiment, FACS analysis was used. (a) Shows the quantification of DNA content in control cells (left panel) and in cells treated with 15 mM aspirin (right panel). Data are represented as cell number (Counts) versus fluorescence intensity (DNA content). The per cent value corresponds to the percentage of apoptotic cells. (b) Shows representative dot plots of Annexin-V-Fluos versus PI fluorescence for control cells (left panel) and cells treated with 15 mM aspirin (right panel). Quadrants: lower left, viable cells; lower right, apoptotic cells; upper right, necrotic cells. Approximately 25 000 cells were analysed for each sample. ASA denotes aspirin.

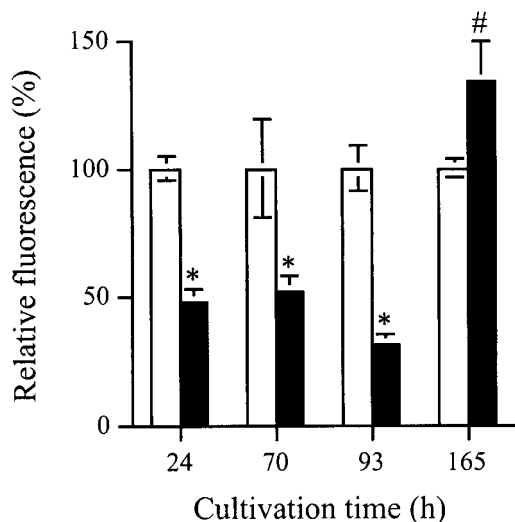


Fig. 5. Intracellular oxidation levels of *S. cerevisiae* EG110 cells (deficient in MnSOD) growing in YPE medium without aspirin (open bars) and in the presence of 15 mM aspirin (solid bars). The oxidant-sensitive probe DCDHF-DA was used. Relative fluorescence was measured at 517 nm with excitation at 490 nm. Background fluorescence was subtracted from the data. Each point represents the mean of at least four independent determinations. Error bars are ± 1 SD and appear where sufficiently large. *, $P < 0.001$; #, $P < 0.01$; treatment versus control, two-tailed *t*-test.

ACKNOWLEDGEMENTS

We thank the reviewers for suggestions to improve the manuscript. We are grateful to Professor Alex Felice for use of the flowcytometer

and Dr Mark Micallef, Mr Godfrey Grech and Ms Wilma Cassar for technical advice on flowcytometry. This work was supported by a Research Fund Grant from the University of Malta.

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