Mitochondrial involvement in aspirin-induced apoptosis in yeast

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We have previously reported that aspirin induces apoptosis in manganese superoxide dismutase (MnSOD)-deficient Saccharomyces cerevisiae cells when cultivated on the non-fermentable carbon source ethanol. Here, we investigated the role of mitochondria in aspirin-induced apoptosis. We report that aspirin had an inhibitory effect on cellular respiration, and caused the release of most of the mitochondrial cytochrome c and a dramatic drop in the mitochondrial membrane potential ($\Delta \Psi_m$). Also, aspirin reduced the intracellular cytosolic pH in the MnSOD-deficient cells growing in ethanol medium, but this did not seem to be the initial trigger that committed these cells to aspirin-induced apoptosis. Furthermore, loss of $\Delta \Psi_m$ was not required for aspirin-induced release of cytochrome c, since the initial release of cytochrome c occurred prior to the disruption of the $\Delta \Psi_m$. It is thus possible that cytochrome c release does not involve the early onset of the mitochondrial permeability transition, but only an alteration of the permeability of the outer mitochondrial membrane.

INTRODUCTION

Non-steroidal anti-inflammatory drugs, principally aspirin, have anti-neoplastic properties, as shown by epidemiological studies on colorectal cancer (Kune et al., 1988; Chan et al., 2004). The chemopreventive and anti-proliferative properties of aspirin towards cell cultures and tumour cells have been shown to be due to the induction of apoptosis (Qiao et al., 1998; Pique et al., 2000). Several molecular mechanisms that underlie the apoptotic effect of aspirin have been reported, including p38 MAP kinase activation in human fibroblasts (Schwenger et al., 1998), inhibition of human telomerase reverse transcriptase in colon cancer cells (He et al., 2005) and alteration of the signalling pathway of the transcription factor NF-κB in human gastric cells (Stark & Dunlop, 2005). Moreover, aspirin enhances tumour apoptotic cell death by promoting the onset of the mitochondrial permeability transition (Uyemura et al., 1997; Oh et al., 2003).

Apoptosis in yeast cells is accompanied by typical features of mammalian apoptosis (Madeo et al., 1997) after treatment of the cells with low doses of hydrogen peroxide (Madeo et al., 1999), z mating-type pheromone (Severin & Hyman, 2002) and hyperosmotic stress (Silva et al., 2005). Also, orthologues of key regulators of mammalian apoptosis, such as the metacaspase Yca1p (Madeo et al., 2002), the serine protease HtrA2/Omi (Fahrenkrog et al., 2004), apoptosis-inducing factor (Aif1p) (Wissing et al., 2004) and mitochondrial endonuclease G (Nuc1p) (Büttner et al., 2007) have been characterized in yeast, thus demonstrating the presence of a basic apoptotic machinery similar to that found in higher organisms. Furthermore, this model system has been used to probe the relation between apoptosis and mitochondrial fragmentation (Fannjiang et al., 2004), histone H2B phosphorylation (Ahn et al., 2005) and tBid/Bax phosphorylation (Büttner et al., 2007).

In previous work, we studied the effect of aspirin on Saccharomyces cerevisiae cells with differential protection against reactive oxygen species (ROS) and differential production of ROS, which was obtained with growth of wild-type and manganese superoxide dismutase (MnSOD)-deficient cells on fermentable and non-fermentable carbon sources. We showed that aspirin-treated MnSOD-deficient yeast cells died through a late apoptotic process when cultivated on the non-fermentable carbon source ethanol. Aspirin was found to act as an antioxidant until the appearance of apoptosis (Balzan et al., 2004). However, aspirin caused an early shift in the redox environment, due to depletion of NADPH and NADP+, which led to a dramatic drop in the GSH/GSSG concentration ratio (Sapienza & Balzan, 2005).
In the present work, we used the same experimental model of apoptosis and investigated the role of mitochondria in aspirin-induced apoptosis in the MnSOD-deficient yeast cells growing in ethanol medium, with a focus on cytochrome c release and the effect of aspirin on the mitochondrial membrane potential ($\Delta \Psi_m$). We also studied the effect of aspirin on the intracellular cytosolic pH ($p_{\text{H}}$) of these cells.

**METHODS**

**Culture conditions and treatments.** The parent *S. cerevisiae* strain used in this study was EG103 (MATa leu2-3 112 his3A1 trpl-289a ura3-52 GAL+) and the MnSOD-deficient strain was EG110 (EG103 sod2A::TRP1), kindly provided by Edith Gralla of University of California, Los Angeles, and Valeria C. Culotta of Johns Hopkins University. Cells were grown in enriched yeast extract, peptone-based medium with 3% (v/v) ethanol. For plates, 2% (w/v) agar was used and incubation was at 28 °C. Aerobic growth in liquid culture was maintained at 28 °C with constant shaking at 250 r.p.m. The cells were also cultured in fresh media in the presence of 15 mM aspirin (acetylsalicylic acid) (Sigma), and the pH of the medium was adjusted to 5.5 with 1 M Trizma base (Sigma). Growth was followed by monitoring OD$_{600}$. Cultures with OD$_{600}$ values greater than 1.0 were diluted as necessary.

**Oxygen consumption.** The cellular oxygen consumption of yeast cells was measured polarographically, as described elsewhere (Longo et al., 1999), using a YSI Biological Oxygen Monitor (model 5300) and a Clark-type oxygen electrode. Briefly, yeast cells (3.2 × 10$^8$) were added to a sample chamber containing 4 ml YPE medium, with or without 15 mM aspirin, and were magnetically stirred at 30 °C. In this way, the rate of oxygen consumption was measured under closely similar conditions to those found in the flask cultures. The oxygen consumption of the yeast cells was monitored after the addition of carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma) and potassium cyanide (KCN) (BDH) to the culture medium. Four micromolar FCCP accelerated respiration and 700 μM KCN inhibited respiration, as expected.

**Isolation of mitochondria and Western blot analysis.** Isolation of the yeast mitochondrial and membrane-free cytosolic fractions was carried out as described by Glick & Poon (1995). Both the mitochondrial and cytosolic extracts were then aliquoted, flash-frozen and stored at −80 °C. Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories), using BSA as protein standard. For Western blot analysis, 10 μg of protein from the mitochondrial fractions and 40 μg of protein from the cytosolic fractions, obtained from aspirin-treated and untreated EG110 cells, were subjected to 15% (w/v) SDS-PAGE and transferred overnight into nitrocellulose membranes (Amersham International). Blots were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h, washed thoroughly with TBS-T, and incubated with the following primary antibodies in TBS-T for 1 h: anti-cytochrome c (Davids Biotechnologie), anti-HPSE60 (kindly provided by G. Schatz, Basel University) and anti-β-actin (Abcam). This was followed by incubation with horseradish peroxidase-labelled secondary antibody, and the immunoreactive bands were detected by enhanced chemiluminescence (ECL Western blotting detection kit, Amersham Biosciences).

**Measurement of $\Delta \Psi_m$ and mitochondrial mass.** The $\Delta \Psi_m$ of yeast cells was determined as described elsewhere (Ludovico et al., 2001), utilizing the fluorescent probe rhodamine 123 (Rh123) (Molecular Probes). Yeast cells (1 × 10$^8$) were harvested, suspended in 1 ml distilled water (pH 6.0) and incubated in 200 mM Rh123 for 30 min at room temperature. The cells were recovered, resuspended in 400 μl distilled water and analysed on the Bio-Rad BRYTE HS flow cytometer. As a positive control, cells were treated with 20 mM sodium azide (Sigma) prior to staining with Rh123. The green fluorescence was gated in a scattergram of log(SS) (side scatter)−log(FS) (forward scatter) in order to include the subpopulation with the highest frequency and homogeneity in the fluorescence measurement. Mitochondrial mass was detected using 10-N-nonyl acridine orange (NAO) (Molecular Probes), as described elsewhere (Massari et al., 2000). Cells (1 × 10$^8$) obtained from the same cell cultures used to measure $\Delta \Psi_m$ were harvested, washed with sterile distilled water and fixed with ice-cold 80% (v/v) ethanol at −20 °C overnight. The cells were recovered, washed twice in distilled water and incubated in 10 μM NAO for 30 min at room temperature. The cells were resuspended in 400 μl sterile distilled water and FACs analysis was carried out using a Bio-Rad BRYTE HS flow cytometer.

**Measurement of intracellular and extracellular pH.** $p_{\text{H}}$ was measured using the ratiometric dye 5-(and-6-)carboxy-semi-naphthorhodafluor-1-acetoxyethyl ester (C-SNARF-1-AM) (Molecular Probes), as described by Haworth et al. (1991), with some modifications. Yeast cells (1 × 10$^8$ ml$^{-1}$) were harvested and incubated with 20 μM C-SNARF-1-AM in 0.2 M Tris-acetate, pH 5.0, for 3 h at 21 °C. An *in vivo* calibration curve was prepared by suspending the C-SNARF-1-AM-loaded cells in 0.2 M acetic acid-Tris/Tris-acetate buffers, over the pH range 4.0–10.0. The cells were then incubated with 30 μM amphotericin B (Sigma) at 30 °C for 1 h, so that the $p_{\text{H}}$ equilibrated with the extracellular pH. The fluorescence of C-SNARF-1-AM was detected using a Bio-Tek Instruments fluorometer with excitation at 514 nm and emission at 575 nm and 610 nm.

For purposes of calibration, the ratio, $R$, of fluorescence emission at 575 and 610 nm, after background subtraction, was determined at unit pH intervals. The Henderson–Hasselbalch equation:

$$p_{\text{H}}=pK^+ + \log[(R_b-R)/(R-R_b)]$$

was used to determine the intracellular $p_{\text{H}}$. $R_b$ and $R_t$ represent the limiting values of the ratio at acidic and basic $p_{\text{H}}$, respectively, and $pK^+$ represents the apparent $pK$ for dissociation of the probe in the intracellular environment. In each experiment, the values of $R_b$, $R_t$ and $pK^+$ were determined by fitting the data to the equation,

$$R=R_b-(R_t-R_b)/(1+10^{pK^+-p_{\text{H}}})$$

by Marquardt’s method of nonlinear least-squares with uniform weights (Press et al., 1996). The basis of this equation is discussed in Opitz et al. (1994). Both test ratios and calibration curves showed appreciable experimental variation in each set of incubation conditions. Because of this variation, replicates of the predicted values of the $p_{\text{H}}$ were pooled, and a 25% trimmed mean of the data was taken as a measure of the best mean $p_{\text{H}}$.

The extracellular $p_{\text{H}}$ of the cell cultures was measured with a PHM 83 AUTOCLG pH meter (Radiometer).

**RESULTS**

**Cellular growth and respiration is dramatically reduced in aspirin-treated MnSOD-deficient *S. cerevisiae* cells in ethanol medium**

MnSOD-deficient EG110 cells cultivated in YPE medium in the presence of 15 mM aspirin showed the pattern of growth described previously for cells undergoing apoptosis.
(Balzan et al., 2004). The cells ceased to grow after 48 h, and at 96 h cultivation the OD$_{600}$ of the cultures was only 10% of that of control cells.

The ability of S. cerevisiae strains EG103 [containing both MnSOD and copper/zinc superoxide dismutase (CuZnSOD)] and EG110 (deficient in MnSOD) to respire on the non-fermentable carbon source ethanol, in the absence and presence of 15 mM aspirin, was assayed polarographically (Fig. 1). The rate of respiration in EG110 cells was maintained at ~29 nmol O$_2$ min$^{-1}$ per 10$^7$ cells ($\pm$ 2.4 SD) during the different stages of growth. Similarly, respiration in EG103 cells was 28 nmol O$_2$ min$^{-1}$ per 10$^7$ cells ($\pm$ 2.4 SD) until 72 h of growth, and decreased to 20.1 nmol O$_2$ min$^{-1}$ per 10$^7$ cells ($\pm$ 3.3 SD) after 96 h of growth. The decrease in respiration observed in wild-type EG103 cells may be accounted for by the fact that the cells reach stationary phase before MnSOD-deficient EG110 cells. Upon treatment with aspirin, respiration in EG110 cells decreased ninefold after 48 h of cultivation, as compared with untreated EG110 cells, and the rate of respiration decreased further after 72 and 96 h of growth (Fig. 1a). Aspirin also had an inhibitory effect on respiration in EG103 cells; however, after 48 h of cultivation there was a 1.3-fold decrease in respiration. The rate of respiration in aspirin-treated EG103 cells after 96 h of cultivation was higher than in aspirin-treated EG110 cells after 48 h of growth (Fig. 1b). Thus, aspirin has a severe inhibitory effect on mitochondrial respiration, as reflected in cellular respiration in MnSOD-deficient cells when grown in ethanol medium, and a less severe inhibitory effect on mitochondrial respiration in wild-type cells.

**Aspirin induces mitochondrial cytochrome c release in MnSOD-deficient cells in ethanol medium**

Next, we examined whether cytochrome c plays a role in aspirin-induced apoptosis in MnSOD-deficient cells. The levels of cytochrome c in mitochondria and in the cytosol from aspirin-treated EG110 cells was detected by Western blot analysis and immunoblotting. After 48 h of growth in YPE medium, the level of mitochondrial cytochrome c in EG110 cells treated with 15 mM aspirin was similar to that detected in untreated control cells (Fig. 2b, c). However, after 72 h of incubation, there was a slight decrease in the level of mitochondrial cytochrome c, and, after 96 h, a dramatic decrease in the level of mitochondrial cytochrome c was observed (Fig. 2c). The cytochrome c released from the mitochondria was detected in the cytosolic fractions (Fig. 2d). In fact, after 48 h of cultivation no cytochrome c was detected in the cytosol; however, the levels of cytochrome c in the cytosol increased after 72 and 96 h of cultivation (Fig. 2d). No cytochrome c was detected in the cytosol of untreated control cells (data not shown). HSP60 was not detected in the cytosolic fractions of EG110 cells untreated and treated with aspirin, indicating that there was no mitochondrial contamination in the cytosol (data not shown). β-Actin was not detected in the mitochondrial fractions, indicating that there was no mitochondrial contamination from the cytosolic fractions (data not shown). Thus, these findings demonstrate that cytochrome c is translocated from mitochondria into the cytosol during aspirin-induced apoptosis.

**Aspirin causes a drastic fall in the mitochondrial membrane potential of MnSOD-deficient cells and this occurs after the release of cytochrome c**

We then asked whether onset of the mitochondrial permeability transition and loss of ΔΨ$_{m}$ were required for the translocation of mitochondrial cytochrome c to the cytosol. We examined whether the ΔΨ$_{m}$ and mitochondrial mass of aspirin-treated and untreated EG110 cells growing in YPE medium were altered by carrying out FACS analysis of Rh123- and NAO-stained cells, respectively. The relative fluorescence emission in Rh123-stained EG110 cells was similar to that detected in wild-type EG103 cells, which had a relative fluorescence emission of 41.1 ± 2.11 and 41.4 ± 3.01 after 72 and 96 h of growth, respectively. In aspirin-treated EG110 cells, the relative fluorescence emis-

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**Fig. 1.** Cellular respiration of (a) S. cerevisiae EG110 cells (deficient in MnSOD) and (b) EG103 cells (containing both MnSOD and CuZnSOD) growing in YPE medium without aspirin (open bars) and in the presence of 15 mM aspirin (solid bars). Oxygen consumption was monitored using a Clark-type electrode. Each point represents the mean of at least four independent determinations. Error bars represent ±1 SD and appear where sufficiently large. *P<0.001; †P<0.01; treatment versus control, two-tailed t test.
sion was similar to that of untreated control cells after 48 and 72 h of cultivation. However, after 96 h of cultivation there was a drastic fall in the relative fluorescence emission from 39.2 ± 1.62 to 20.3 ± 0.62 (Fig. 3). In fact, the ΔΨm in aspirin-treated MnSOD-deficient cells decreased to nearly half that of untreated EG110 cells.

The mitochondrial mass in aspirin-treated EG110 cells displayed fluorescence comparable to that of untreated control cells. After 48 h of cultivation, the relative fluorescence emission of EG110 cells was 130.6 ± 3.89 and 130.7 ± 4.20 in the absence and presence of aspirin, respectively. After 72 h of growth, the relative fluorescence emission increased to 144.8 ± 4.26 and 141.0 ± 3.26, in the absence and presence of aspirin, respectively, and this remained the same after 96 h of growth. Thus, the mitochondrial mass of aspirin-treated MnSOD-deficient cells was similar to that of untreated cells, indicating that the drastic fall in the ΔΨm of aspirin-treated cells after 96 h of cultivation was not due to a decrease in mitochondrial mass.

**MnSOD-deficient cells are unable to maintain intracellular pH in YPE medium in the presence of aspirin**

The maintenance of cytoplasmic pH is crucial for many enzyme activities and for cellular metabolism, and we next

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**Fig. 2.** Cytochrome c is released from mitochondria into the cytosol during aspirin-induced apoptosis in MnSOD-deficient *S. cerevisiae* EG110 cells growing in YPE medium. Cytochrome c was detected by Western blot analysis and immunoscreening of the mitochondrial and cytosolic fractions, obtained from EG110 cells cultivated in YPE medium, with and without 15 mM aspirin. An equal amount of total mitochondrial protein was loaded in each lane, as demonstrated (a) by HSP60 concentration in the mitochondrial fractions. (b) The level of mitochondrial cytochrome c in EG110 cells, not treated with aspirin, was the same after 48, 72 and 96 h of growth. (c) The level of mitochondrial cytochrome c decreased in aspirin-treated EG110 cells, after 72 and 96 h of growth. (d) Cytochrome c was detected in the cytoplasm after 72 h. After 96 h, most of the mitochondrial cytochrome c was present in the cytosol. (e) β-Actin concentration in the cytosolic fractions. The data represent at least three independent experiments.

**Fig. 3.** Disruption of ΔΨm in aspirin-treated *S. cerevisiae* EG110 cells (deficient in MnSOD). EG110 cells were cultivated in YPE medium in the presence of 15 mM aspirin, pH 5.5, and stained with Rh123 after (a) 48 h, (b) 72 h and (c) 96 h of cultivation. (d) As a positive control, aspirin-treated EG110 cells were treated with 20 mM sodium azide (to disrupt the mitochondrial electron transport chain), prior to Rh123 staining. FACS analysis was used and the data represent at least three independent experiments. Data are represented as cell number (Counts) versus relative fluorescence emission (ΔΨm). Approximately 20 000 cells were analysed for each sample.
assessed whether aspirin altered the intracellular pH, thereby committing MnSOD-deficient cells to apoptosis. The pH$_i$ in the MnSOD-deficient EG110 cells in YPE medium was maintained at ~pH 7.2 during the different phases of growth (Fig. 4a). Aspirin-treated EG110 cells also maintained a pH$_i$ of 7.2 after 48 and 72 h of cultivation. However, after 96 h of growth there was a drop to pH$_i$ 6.5. Statistical analysis of the data showed a significant difference in intracellular pH ($P<0.001$) between aspirin-treated and untreated cells after 96 h of cultivation ($t$ test; see Fig. 4a). This finding indicated that the cells were unable to regulate the cytosolic pH when treated with aspirin. Changes in the external pH of the cell culture corresponded with loss of cellular ability to regulate the cytosolic pH. In fact, the external pH of YPE medium harbouring EG110 cells with or without 15 mM aspirin was initially 5.5, but dropped to 4.95 and 4.88, respectively, after 48 h of cultivation. At 72 h of cultivation, the extracellular pH of EG110 cells treated with aspirin decreased to pH 4.6, and remained the same after 96 h of cultivation. However, the external pH of EG110 cells cultured without aspirin decreased significantly to pH 4.35 ($P<0.05$; $t$ test). There was no significant difference in the pH$_i$ of wild-type EG103 cells in YPE medium with or without aspirin (Fig. 4b). It appears that wild-type cells are able to maintain the pH$_i$ above pH 7.0 throughout the different phases of growth, even in the presence of 15 mM aspirin, whereas aspirin-treated MnSOD-deficient cells are unable to maintain a homeostatic cytosolic pH.

**DISCUSSION**

Our data showed that aspirin inhibits cellular respiration in MnSOD-deficient *S. cerevisiae* cells when cultivated on the non-fermentable carbon source ethanol. The pronounced fall in the rate of respiration was seen after 48 h of growth (Fig. 1a), when the MnSOD-deficient cells were still viable, as measured by the ability to form new colonies. Afterwards these cells undergo a drastic fall in viability in the presence of aspirin (Balzan et al., 2004), which correlates with the observed decrease in respiration. Aspirin also had an inhibitory effect on cellular respiration in wild-type cells (Fig. 1b); however, this was not so severe and did not effect cellular growth or viability (data not shown). Aspirin has been reported to uncouple oxidative phosphorylation in isolated rat liver mitochondria when present in micromolar concentrations (Adams & Cobb, 1958; Somasundaram et al., 1997); however, low millimolar concentrations of aspirin inhibit mitochondrial respiration in isolated rat liver (Somasundaram et al., 1997) and cardiac mitochondria (Nulton-Persson et al., 2004). The inhibitory effect of aspirin on respiration is due to inhibition of the electron transport chain (at complex I and complex II) in isolated rat liver mitochondria (Somasundaram et al., 1997). Nulton-Persson et al. (2004) reported that aspirin limits the supply of NADH to the electron transport chain through inhibition of α-ketoglutarate dehydrogenase in isolated rat cardiac mitochondria. We have previously shown that aspirin significantly decreases the levels of NADP$^+$ and NADPH in MnSOD-deficient yeast cells after 72 h of cultivation in ethanol medium (Sapienza & Balzan, 2005).

The role of cytochrome *c* in aspirin-induced apoptosis in the MnSOD-deficient cells was investigated next, since cytochrome *c* plays a central role in the generation of downstream apoptotic events in mammalian cells (Li et al., 1997). An initial release of cytochrome *c* was detected in the cytosol of aspirin-treated MnSOD-deficient cells, at which time aspirin had no effect on $\Delta \Psi_m$ (Figs 2 and 3). However, after 96 h of cultivation, most of the mitochondrial cytochrome *c* was detected in the cytosol, and this correlated with a dramatic drop in $\Delta \Psi_m$, which decreased to nearly half that of untreated control cells. The results...
confirm our previous finding that aspirin induces apoptosis in the MnSOD-deficient cells grown in ethanol medium (Balzan et al., 2004), with the same time order of apoptosis-related events. Also, the findings are consistent with the results of Pique et al. (2000), whereby aspirin induces apoptosis through mitochondrial cytochrome c release prior to caspase activation and loss of ΔΨm in Jurkat and acute T-leukaemia cell lines. Indeed, the participation of cytochrome c in yeast apoptosis has been suggested, since mutations in cytochrome c haem lyase partially rescue acetic acid-induced cell death (Ludovico et al., 2002) and yeast mutants lacking c-type cytochromes survive after treatment with α-factor and amiodarone (Pozniakovsky et al., 2005).

Our observations demonstrate that loss of ΔΨm is not required for aspirin-induced release of cytochrome c. In fact, the initial release of cytochrome c occurred prior to the disruption of the ΔΨm. It is thus possible that cytochrome c release does not involve the early onset of the mitochondrial permeability transition but only an alteration of the permeability of the outer mitochondrial membrane. Other studies support this, inasmuch as the mitochondrial inner membrane remains intact even under circumstances in which cytochrome c has been released (Priault et al., 1999; Waterhouse et al., 2001).

Expression of Bax in S. cerevisiae, as well as treatment of yeast cells with acetic acid, amiodarone and α-factor, induces a transient increase in the mitochondrial membrane potential, causing mitochondrial ROS formation, followed by depolarization and cell death (Manon et al., 1997; Ludovico et al., 2002; Pozniakovsky et al., 2005). We detected a fall in the ΔΨm at 96 h of cultivation (Fig. 3), at which time the antioxidant effect of aspirin was at its highest (Balzan et al., 2004). Votyakova & Reynolds (2001) have also shown that depolarization of rat brain mitochondria causes a profound reduction in ROS formation. Indeed, Starkov & Fiskum (2003) have demonstrated that ROS production is influenced by ΔΨm and the NAD(P)H redox state in isolated brain mitochondria, in which a reduction in ΔΨm is accompanied by a decrease in H2O2 production in the presence of NADH-linked oxidizable substrates.

Maintenance of a homeostatic pHi is also essential for the proper functioning of enzymes. In fact, the pHi in mammalian cells is maintained around neutrality in order to sustain metabolic pathways (Madshus, 1988). In our study, the mean pHi of MnSOD-deficient cells cultivated in ethanol medium was 7.2 during the different phases of growth, and that in wild-type cells was maintained above 7.0 (Fig. 4a, b). These results agree to some extent with those of other reports. The pHi of a respiratory-deficient S. cerevisiae mutant, IGC3507 III, measured by the distribution of [14C]propanionic acid, was found to be between 7.0 and 7.2 (Pampulha & Loureiro-Dias, 1989). The mean resting internal pH was 6.6 in S. cerevisiae and 7.0 in Schizosaccharomyces pombe, measured using the fluorescent probe C-SNARF-1 (Haworth & Fliegel, 1993).

Although the MnSOD-deficient cells maintained the pH at 7.2 after 72 h of cultivation in the presence of aspirin, after 96 h of cultivation, the pHi fell to 6.5, which is 0.7 pH units lower than in control cells (Fig. 4a). These findings indicate that the MnSOD-deficient cells were unable to maintain the homeostatic cytosolic pH. A decrease in pHi of ~0.3–0.4 units has been detected elsewhere following exposure of mammalian cells to several apoptotic triggers, including UV irradiation, staurosporine, anti-Fas antibodies and growth-factor deprivation (reviewed by Matsuyma & Reed, 2000). An early decrease in the cytosolic pH was detected after treatment of Jurkat cells with the kinase inhibitor staurosporine. Our results suggest that the imbalance in pHi regulation, as reflected by the fall in the cytosolic pH of aspirin-treated MnSOD-deficient cells, may reflect compromised energy stores and commitment to apoptosis. Several reports show that the mechanism of pH homeostasis is dependent on the presence of an energy source (Karagiannis & Young, 2001; Piper et al., 2001). However, the fall in pHi in the MnSOD-deficient strain does not seem to be the initial trigger that commits these cells to apoptosis, since it occurred only after 96 h of cultivation, when the cells would have already lost their viability.

Our findings suggest that inhibition of aerobic respiration in the presence of aspirin is linked to the development of apoptosis in MnSOD-deficient yeast cells grown on ethanol medium, without implying direct causation. Additionally, this inhibitory effect of aspirin may account for the low ROS levels detected in the yeast cells (Balzan et al., 2004). The presence of MnSOD in isogenic wild-type cells seems to have a protective effect on mitochondria, since aerobic respiration is not inhibited as extensively as in MnSOD-deficient cells. A protective effect of MnSOD on complexes I and III of the respiratory chain has been observed in 32D cl 3 haematopoietic cells overexpressing a human MnSOD (SOD2) transgene when exposed to radiation (Pearce et al., 2001). In our work, inhibition of aerobic respiration and concomitant redox imbalance (Sapienza & Balzan, 2005) precede apoptotic induction. This possibly caused the initial release of mitochondrial cytochrome c, followed by disruption of the ΔΨm, further release of nearly all the mitochondrial cytochrome c and concomitant decrease in pHi. Fig. 5 summarizes the sequence of events observed to lead to apoptosis. We propose to investigate the mitochondrial origin of the apoptotic process and its relationship to inhibition of aerobic respiration on ethanol medium by cloning and targeting of exogenous MnSOD to the mitochondria of the yeast cells.

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The initial release of mitochondrial cytochrome c preceded by loss of viability and occurred after 96 h of cultivation. Inhibition of aerobic respiration occurred at 48 h of cultivation when the cells were still viable. Apoptosis was associated with the release of cytochrome c, decrease of mitochondrial membrane potential, further release of cytochrome c, and decrease of cytosolic pH. Decrease of mitochondrial membrane potential may be due to an alteration of the permeability of the outer mitochondrial membrane rather than onset of the mitochondrial permeability transition (MPT), because it occurs before the decrease in the ΔΨm. MPT may occur afterwards when there is a decrease of ΔΨm and further release of cytochrome c. Decrease of the intracellular cytosolic pH also occurs at the same time.

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