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.

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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.*, 2006), activation of caspases (Castano *et al.*, 1999; Power *et al.*, 2004), downregulation of the anti-apoptotic Bcl-2 protein (Gao *et al.*, 2004; Redlak *et al.*, 2005) and alteration of the signalling pathway of the transcription factor NF- κ B 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), α 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-induced mitochondrial cytochrome *c* release (Ott *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).

Abbreviations: C-SNARF-1-AM, 5-(and-6)-carboxy-seminaphthorhodafuor-1-acetoxymethyl ester; CuZnSOD, copper/zinc superoxide dismutase; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; $\Delta\Psi_m$, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; NAO, 10-*N*-nonyl acridine orange; pH_i, intracellular pH; Rh123, rhodamine 123; ROS, reactive oxygen species.

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 (pH_i) of these cells.

METHODS

Culture conditions and treatments. The parent *S. cerevisiae* strain used in this study was EG103 (*MAT α leu2-3 112 his3 Δ 1 trp1-289a ura3-52 GAL⁺*) and the MnSOD-deficient strain was EG110 (EG103 *sod2 Δ ::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 OD₆₀₀. Cultures with OD₆₀₀ 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 & Pon (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 onto 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-HSP60 (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 (1×10^6) were harvested, suspended in 1 ml distilled water (pH 6.0) and incubated in 200 nM 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 (1×10^6) 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. pH_i was measured using the ratiometric dye 5-(and-6)-carboxy-semi-naphthorhodafluor-1-acetoxymethyl ester (C-SNARF-1-AM) (Molecular Probes), as described by Haworth *et al.* (1991), with some modifications. Yeast cells ($1 \times 10^8 \text{ ml}^{-1}$) 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 pH_i 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:

$$\text{pH} = \text{pK}' + \log[(R_a - R)/(R - R_b)]$$

was used to determine the intracellular pH. R_a and R_b represent the limiting values of the ratio at acidic and basic pH, respectively, and pK' represents the apparent pK for dissociation of the probe in the intracellular environment. In each experiment, the values of R_a , R_b and pK' were determined by fitting the data to the equation,

$$R = R_a - (R_a - R_b)/(1 + 10^{\text{pK}' - \text{pH}})$$

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 pH_i were pooled, and a 25% trimmed mean of the data was taken as a measure of the best mean pH.

The extracellular pH of the cell cultures was measured with a PHM 83 AUTOCAL 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 OD₆₀₀ 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 O₂ min⁻¹ per 10⁷ cells (± 2.4 SD) during the different stages of growth. Similarly, respiration in EG103 cells was 28 nmol O₂ min⁻¹ per 10⁷ cells (± 2.4 SD) until 72 h of growth, and decreased to 20.1 nmol O₂ min⁻¹ per 10⁷ cells (± 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 immunoscreening. 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 cultivation, 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 $\Delta\Psi_m$ were required for the translocation of mitochondrial cytochrome *c* to the cytosol. We examined whether the $\Delta\Psi_m$ 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-

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.

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 cytosol 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.

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 $\Delta\Psi_m$ 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

Fig. 3. Disruption of $\Delta\Psi_m$ 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 ($\Delta\Psi_m$). Approximately 20 000 cells were analysed for each sample.

drastic fall in the $\Delta\Psi_m$ 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

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 $\sim pH$ 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

Fig. 4. Intracellular pH 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). The ratiometric fluorescent dye C-SNARF-1-AM was used. The emission spectra were measured at 575 and 610 nm, with excitation at 514 nm. Background fluorescence was subtracted from the data. Each point represents the mean of at least four independent determinations. Error bars, ± 1 SD; * $P < 0.001$; treatment versus control, two-tailed t test.

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 $\Delta\Psi_m$ 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 $\Delta\Psi_m$ 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 $\Delta\Psi_m$. 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. 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 $\Delta\Psi_m$ 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 $\Delta\Psi_m$ and the NAD(P)H redox state in isolated brain mitochondria, in which a reduction in $\Delta\Psi_m$ is accompanied by a decrease in H_2O_2 production in the presence of NADH-linked oxidizable substrates.

Maintenance of a homeostatic pH_i is also essential for the proper functioning of enzymes. In fact, the pH_i in mammalian cells is maintained around neutrality in order to sustain metabolic pathways (Madshus, 1988). In our study, the mean pH_i 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 pH_i of a respiratory-deficient *S. cerevisiae* mutant, IGC3507 III, measured by the distribution of [^{14}C]propionic 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_i at 7.2 after 72 h of cultivation in the presence of aspirin, after 96 h of cultivation, the pH_i 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 pH_i 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 Matsuyama & 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 pH_i 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 pH_i 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 $\Delta\Psi_m$, further release of nearly all the mitochondrial cytochrome *c* and concomitant decrease in pH_i . 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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Fig. 5. Sequence of events associated with apoptosis in MnSOD-deficient yeast cells grown on ethanol medium in the presence of aspirin. Inhibition of aerobic respiration occurred at 48 h of cultivation when the cells were still viable. Apoptosis was preceded by loss of viability and occurred after 96 h of cultivation. The initial release of mitochondrial cytochrome *c* 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 $\Delta\Psi_m$. MPT may occur afterwards when there is a decrease of $\Delta\Psi_m$ and further release of cytochrome *c*. Decrease of the intracellular cytosolic pH also occurs at the same time.

REFERENCES

- Adams, S. S. & Cobb, R. (1958). A possible basis for the anti-inflammatory activity of salicylates and other non-hormonal anti-rheumatic drugs. *Nature* **181**, 773–774.
- Ahn, S. H., Cheung, W. L., Hsu, J. Y., Diaz, R. L., Smith, M. M. & Allis, C. D. (2005). Sterile 20 kinase phosphorylates histone H2B at serine 10 during hydrogen peroxide-induced apoptosis in *S. cerevisiae*. *Cell* **120**, 25–36.

- Balzan, R., Sapienza, K., Galea, D. R., Vassallo, N., Frey, H. & Bannister, W. H. (2004). Aspirin commits yeast cells to apoptosis depending on carbon source. *Microbiology* **150**, 109–115.
- Büttner, S., Eisenberg, T., Carmona-Gutierrez, D., Ruli, D., Knauer, H., Ruckenstuhl, C., Sigrist, C., Wissing, S., Kollrosier, M. & other authors (2007). Endonuclease G regulates budding yeast life and death. *Mol Cell* **25**, 233–246.
- Castano, E., Dalmau, M., Barragan, M., Pueyo, G., Bartrons, R. & Gil, J. (1999). Aspirin induces cell death and caspase-dependent phosphatidylserine externalization in HT-29 human colon adenocarcinoma cells. *Br J Cancer* **81**, 294–299.
- Chan, A. T., Giovannucci, E. L., Schernhammer, E. S., Colditz, G. A., Hunter, D. J., Willett, W. C. & Fuch, C. S. (2004). A prospective study of aspirin use and the risk for colorectal adenoma. *Ann Intern Med* **140**, 157–166.
- Fahrenkrog, B., Sauder, U. & Aebi, U. (2004). The *S. cerevisiae* HtrA-like protein Nma11p is a nuclear serine protease that mediates yeast apoptosis. *J Cell Sci* **117**, 115–126.
- Fannjiang, Y., Cheng, W. C., Lee, S. J., Qi, B., Pevsner, J., McCaffery, J. M., Hill, R. B., Basanez, G. & Hardwick, J. M. (2004). Mitochondrial fission proteins regulate programmed cell death in yeast. *Genes Dev* **18**, 2785–2797.
- Gao, J., Niwa, K., Sun, W., Takemura, M., Lian, Z., Onogi, K., Seishima, M., Mori, H. & Tamaya, T. (2004). Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and up-regulate cyclooxygenase-2 protein expression in endometrial cancer cells. *Cancer Sci* **95**, 901–907.
- Glick, B. S. & Pon, L. A. (1995). Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. *Methods Enzymol* **260**, 213–223.
- Haworth, R. S. & Fliegel, L. (1993). Intracellular pH in *Schizosaccharomyces pombe* – comparison with *Saccharomyces cerevisiae*. *Mol Cell Biochem* **124**, 131–140.
- Haworth, R. S., Lemire, B. D., Crandall, D., Cragoe, E. J., Jr & Fliegel, L. (1991). Characterisation of proton fluxes across the cytoplasmic membrane of the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1098**, 79–89.
- He, H., Xia, H. H., Wang, J. D., Gu, Q., Lin, M. C., Zou, B., Lam, S. K., Chan, A. O., Yuen, M. F. & other authors (2006). Inhibition of human telomerase reverse transcriptase by nonsteroidal antiinflammatory drugs in colon carcinoma. *Cancer* **106**, 1243–1249.
- Karagiannis, J. & Young, P. G. (2001). Intracellular pH homeostasis during cell-cycle progression and growth state transition in *Schizosaccharomyces pombe*. *J Cell Sci* **114**, 2929–2941.
- Kune, G. A., Kune, S. & Watson, L. F. (1988). Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the Melbourne Colorectal Cancer Study. *Cancer Res* **48**, 4399–4404.
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. & Wang, X. (1997). Cytochrome *c* release and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489.
- Longo, V. D., Liou, L. L., Valentine, J. S. & Gralla, E. B. (1999). Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch Biochem Biophys* **365**, 131–142.
- Ludovico, P., Sansonetti, F. & Côte-Real, M. (2001). Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology* **147**, 3335–3343.
- Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A. & Côte-Real, M. (2002). Cytochrome *c* release and mitochondria involvement in programmed cell death induced by acetic acid in *Saccharomyces cerevisiae*. *Mol Biol Cell* **13**, 2598–2606.

- Madeo, F., Frohlich, E. & Frohlich, K.-U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. *J Cell Biol* 139, 729–734.
- Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H. & Frohlich, K.-U. (1999). Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* 145, 757–767.
- Madeo, F., Herker, E., Maldener, C., Wissing, S., Lächelt, S., Herlan, M., Fehr, M., Lauber, K., Sigrist, S. J. & other authors (2002). A caspase-related protease regulates apoptosis in yeast. *Mol Cell* 9, 911–917.
- Madhus, I. H. (1988). Regulation of intracellular pH in eukaryotic cells. *Biochem J* 250, 1–8.
- Manon, S., Chaudhuri, B. & Guerin, M. (1997). Release of cytochrome *c* and decrease of cytochrome *c* oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-x_L. *FEBS Lett* 415, 29–32.
- Massari, P., Ho, Y. & Wetzler, L. M. (2000). *Neisseria meningitidis* porin PorB interacts with mitochondria and protects cells from apoptosis. *Proc Natl Acad Sci U S A* 97, 9070–9075.
- Matsuyama, S. & Reed, J. C. (2000). Mitochondria-dependent apoptosis and cellular pH regulation. *Cell Death Differ* 7, 1155–1165.
- Nulton-Persson, A. C., Szveda, L. I. & Sadek, H. A. (2004). Inhibition of cardiac mitochondrial respiration by salicylic acid and acetylsalicylate. *J Cardiovasc Pharmacol* 44, 591–595.
- Oh, K. W., Qian, T., Brenner, D. A. & Lemasters, J. J. (2003). Salicylate enhances necrosis and apoptosis mediated by the mitochondrial permeability transition. *Toxicol Sci* 73, 44–52.
- Opitz, N., Merten, E. & Acker, H. (1994). Evidence for redistribution-associated intracellular pK' shifts of the pH-sensitive fluorophore carboxy-SNARF-1. *Pflügers Arch* 427, 332–342.
- Ott, M., Norberg, E., Walter, K. M., Schreiner, P., Kemper, C., Rapaport, D., Zhivotovsky, B. & Orrenius, S. (2007). The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome *c* release. *J Biol Chem* 282, 27633–27639.
- Pampulha, M. E. & Loureiro-Dias, M. C. (1989). Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Appl Microbiol Biotechnol* 31, 547–550.
- Pearce, L. L., Epperly, M. W., Greenberger, J. S., Pitt, B. R. & Peterson, J. (2001). Identification of respiratory complexes I and III as mitochondrial sites of damage following exposure to ionizing radiation and nitric oxide. *Nitric Oxide* 5, 128–136.
- Piper, P., Calderon, C. O., Hatzixanthis, K. & Mollapour, M. (2001). Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* 147, 2635–2642.
- Pique, M., Barragan, M., Dalmau, M., Bellosillo, B., Pons, G. & Gil, J. (2000). Aspirin induces apoptosis through mitochondrial cytochrome *c* release. *FEBS Lett* 480, 193–196.
- Power, J. J., Dennis, M. S., Redlak, M. J. & Miller, T. A. (2004). Aspirin-induced mucosal cell death in human gastric cells: evidence supporting an apoptotic mechanism. *Dig Dis Sci* 49, 1518–1525.
- Pozniakovsky, A. I., Knorre, D. A., Markova, O. V., Hyman, A. A., Skulachev, V. P. & Severin, F. F. (2005). Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. *J Cell Biol* 168, 257–269.
- Press, W. H., Teukolsky, S. A., Vetterling, W. T. & Flannery, B. P. (1996). *Numerical Recipes in C*, 2nd edn. Cambridge: Cambridge University Press.
- Priault, M., Chaudhuri, B., Clow, A., Camougrand, N. & Manon, S. (1999). Investigation of bax-induced release of cytochrome *c* from yeast mitochondria permeability of mitochondrial membranes, role of VDAC and ATP requirement. *Eur J Biochem* 260, 684–691.
- Qiao, L., Hanif, R., Sphicas, E., Shiff, S. J. & Rigas, B. (1998). Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. *Biochem Pharmacol* 55, 53–64.
- Redlak, M. J., Power, J. J. & Miller, T. A. (2005). Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 289, G731–G738.
- Sapienza, K. & Balzan, R. (2005). Metabolic aspects of aspirin-induced apoptosis in yeast. *FEMS Yeast Res* 5, 1207–1213.
- Schwenger, P., Alpert, D., Skolnik, E. Y. & Vilcek, J. (1998). Activation of p38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IκB phosphorylation and degradation. *Mol Cell Biol* 18, 78–84.
- Severin, F. F. & Hyman, A. A. (2002). Pheromone induces programmed cell death in *S. cerevisiae*. *Curr Biol* 12, R233–R235.
- Silva, R. D., Sotoca, R., Johansson, B., Ludovico, P., Sansonetty, F., Silva, M. T., Peinado, J. M. & Côrte-Real, M. (2005). Hyperosmotic stress induces metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae*. *Mol Microbiol* 58, 824–834.
- Somasundaram, S., Rafi, S., Hayllar, J., Sigthorsson, G., Jacob, M., Price, A. B., Macpherson, A., Mahmood, T., Scott, D. & other authors (1997). Mitochondrial damage: a possible mechanism of the “topical” phase of NSAID induced injury to the rat intestine. *Gut* 41, 344–353.
- Stark, L. A. & Dunlop, M. G. (2005). Nucleolar sequestration of RelA (p65) regulates NF-κB-driven transcription and apoptosis. *Mol Cell Biol* 25, 5985–6004.
- Starkov, A. A. & Fiskum, G. (2003). Regulation of brain mitochondrial H₂O₂ production by membrane potential and NAD(P)H redox state. *J Neurochem* 86, 1101–1107.
- Uyemura, S. A., Santos, A. C., Mingatto, F. E., Jordani, M. C. & Curti, C. (1997). Diclofenac sodium and mefenamic acid: potent inducers of the membrane permeability transition in renal cortex mitochondria. *Arch Biochem Biophys* 342, 231–235.
- Votyakova, T. V. & Reynolds, I. J. (2001). ΔΨ_m-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79, 266–277.
- Waterhouse, N. J., Goldstein, J. C., von Ahsen, O., Schuler, M., Newmeyer, D. D. & Green, D. R. (2001). Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J Cell Biol* 153, 319–328.
- Wissing, S., Ludovico, P., Herker, E., Büttner, S., Engelhardt, S. M., Decker, T., Link, A., Proksch, A., Rodrigues, F. & other authors (2004). An AIF orthologue regulates apoptosis in yeast. *J Cell Biol* 166, 969–974.

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