RESEARCH ARTICLE

Aspirin-induced apoptosis of yeast cells is associated with mitochondrial superoxide radical accumulation and NAD(P)H oxidation

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Abstract

In previous studies, we observed that aspirin, a promising cancer-preventive agent, induces apoptosis in mitochondrial manganese superoxide dismutase (MnSOD)-deficient *Saccharomyces cerevisiae* cells grown aerobically in ethanol medium. In this study, we show that aspirin-induced apoptosis is associated with a significant increase in mitochondrial and cytosolic $O_2^{\cdot-}$ and oxidation of mitochondrial NAD(P)H. A concomitant rise in the level of cytosolic CuZn-SOD activity failed to compensate for mitochondrial MnSOD deficiency. However, an observed increase in activity of *Escherichia coli* FeSOD targeted to the mitochondrial matrix of the MnSOD-deficient yeast cells, markedly decreased aspirin-induced accumulation of mitochondrial $O_2^{\cdot-}$, significantly increased the mitochondrial NAD(P)H level and rescued the apoptotic phenotype. Indeed, recombinant yeast cells expressing *E. coli* FeSOD behaved in a similar manner to the parent wild-type yeast cells with native mitochondrial MnSOD activity. Wild-type cells consistently showed a decrease in mitochondrial $O_2^{\cdot-}$ and an increase in mitochondrial NAD(P)H levels in the presence of aspirin in ethanol medium. In fact, in wild-type cells, our studies supported an antioxidant action of aspirin. Taken together, our results indicate that a pro-oxidant effect of aspirin occurring predominantly in cells with compromised mitochondrial redox balance may be enough to overcome antioxidant defences resulting in apoptosis, as observed in MnSOD-deficient yeast cells.

Introduction

In addition to the well-established pharmacological actions of the nonsteroidal anti-inflammatory drug aspirin (acetylsalicylic acid), there is broad recognition of its chemopreventive properties (Rothwell *et al.*, 2011). This has been attributed to its ability to induce apoptosis (Qiao *et al.*, 1998). Yeast cells are among the experimental models used extensively for the study of oxidative stress and apoptosis in living organisms because yeast, such as *S. cerevisiae*, retains many of the core eukaryotic cellular processes, including the hallmarks of eukaryotic apoptosis (Madeo *et al.*, 1997; Carmona-Gutierrez *et al.*, 2010; Farrugia & Balzan, 2012).

Aspirin has been observed to act as a pro-oxidant molecule that causes apoptosis by increasing the production of reactive oxygen species (ROS) (Raza *et al.*, 2011). This is consonant with the concept that oxidative stress, however produced, regulates the level of apoptosis in yeast (Madeo *et al.*, 1999). On the other hand, there is also evidence in support of aspirin acting as a cytoprotective antioxidant by scavenging of ROS (Yiannakopoulou & Tiligada, 2009; Baltazar *et al.*, 2011). This dichotomous nature of aspirin as both pro-oxidant and antioxidant is reflected in various conflicting reports on effects of aspirin on the superoxide dismutases (SODs) and other antioxidant enzymes. For example, aspirin was shown to reduce the levels of antioxidant enzymes, such as SODs, in rat intestine (Nair *et al.*, 2006). However, the opposite trend was observed in studies of the effect of aspirin on *Caenorhabditis elegans* exposed to H_2O_2 (Ayyadevara *et al.*, 2013).

In previous work, we studied the effect of aspirin on *S. cerevisiae* cells with differential protection against ROS

and differential production of these species. We observed that yeast cells deficient in mitochondrial manganese superoxide dismutase (MnSOD) and cultivated in ethanol medium died through apoptosis when treated with aspirin (Balzan *et al.*, 2004). In fact, in these cells, aspirin caused the release of most of the mitochondrial cytochrome *c* and a marked drop in the mitochondrial membrane potential ($\Delta\Psi_m$) (Sapienza *et al.*, 2008), as observed in acetic acid-induced apoptosis (Ludovico *et al.*, 2002). Under the same growth conditions, copper, zinc superoxide dismutase (CuZnSOD)-deficient and wild-type cells remained viable (Balzan *et al.*, 2004).

In this continuation of our work, our main objective was to further investigate mitochondrial involvement in aspirin-induced apoptosis in yeast cells in terms of the critical cytoprotective role of mitochondrial SOD and the potential aspirin-induced generation of mitochondrial ROS, considering the controversial pro- vs antioxidant effects of aspirin in living organisms. We then examined the correlation of the observed accumulation of mitochondrial superoxide ($O_2^{\cdot-}$) induced by aspirin in the absence of mitochondrial SOD with the possible oxidation of mitochondrial NAD(P)H.

Materials and methods

Strains and plasmids

The yeast strains used in this study were the wild-type EG103 (*MATα leu2-3 112 his3Δ1 trp1-289a ura3-52 GAL*$^+$) and the MnSOD-deficient yeast strain EG110 (EG103 *sod2 Δ:: TRP1*), kindly provided by Edith Gralla, University of California, Los Angeles and Valeria C. Culotta, Johns Hopkins University, Baltimore. The plasmids used were YEp/PGK kindly provided by S. Oliver, Manchester University and YEp/PGK-S, which contains the *E. coli* FeSOD gene with the yeast MnSOD gene leader sequence encoding the yeast MnSOD mitochondrial targeting signal (Balzan *et al.*, 1995).

Transformation of yeast cells and fluorimetric analysis of aspirin-induced apoptosis

Transformation of the MnSOD-deficient *S. cerevisiae* EG110 cells by the recombinant plasmids YEp/PGK and YEp/PGK-S was performed by the lithium acetate method (Ito *et al.*, 1983).

For the fluorimetric detection of apoptosis in yeast cells, the preparation and double staining of *S. cerevisiae* cells with FITC-Annexin-V and propidium iodide (PI; Vybrant Apoptosis Assay Kit #3; Invitrogen Molecular Probes, Oregon) were carried out according to Madeo *et al.* (1997), with some modifications. FITC-Annexin-V binds to phosphatidylserine molecules externalized on the cell surface membrane of apoptotic cells and confers on them a green fluorescence (Koopman *et al.*, 1994). PI, a red fluorescent nucleic acid-binding dye, can only bind to the nucleic acids of dead cells that have a ruptured cell surface membrane (Moore *et al.*, 1998).

After the required time of aerobic growth in 250 mL of YPE medium with or without 15 mM aspirin, 1×10^7 yeast cells were harvested and washed in 1 mL of phosphate buffer (pH 6.8) with 1.2 M sorbitol and 0.5 mM $MgCl_2$. The cells were then pelleted by centrifugation (3000 ***g***, 5 min) at room temperature and resuspended in 1 mL of Tris DTT buffer (pH 9.4), with gentle shaking for 15 min at 30 °C. The cells were pelleted by centrifugation (3000 ***g***, 5 min) at room temperature and washed once with 1 mL of phosphosphate buffer (pH 6.8) containing sorbitol. The cells were spun down again, and the wet weight of the cell pellet was recorded. For digestion of the cell wall, the cells were resuspended in 0.5 mL of phosphate buffer containing sorbitol and 20 mg of Zymolase 20T (AMS Biotechnology Ltd, (Europe), UK) per gram wet weight of the cells. The cell suspension was incubated for 2 h at 30 °C with gentle shaking. The spheroplasts were then pelleted by centrifugation (1500 ***g***, 5 min) and washed with 1 mL of 1x annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ in distilled water at pH 7.4) containing 1.2 M Sorbitol, pH 7. 4. The cells were spun down again and resuspended in 1 mL of 1x annexin-binding buffer containing 1.2 M sorbitol, pH 7.4. From this cell suspension, two 0.45 mL volumes were withdrawn and spun down separately into two equal-sized cell pellets, one of which was resuspended in 400 μL of 1x annexin-binding buffer containing 1.2 M sorbitol, pH 7.4, whilst the other was resuspended in 400 μL 1x annexin-binding buffer containing 1.2 M sorbitol, pH 7.4, plus 20 μL of FITC-Annexin-V and 5 μL of PI. Both the unstained (blank) and double-stained cell suspensions were incubated away from light for 30 min at room temperature with gentle shaking, after which they were separately washed with 0.5 mL of 1x annexin-binding buffer without sorbitol. The unstained and stained cell pellets were each resuspended in 0.45 mL of 1x annexin-binding buffer without sorbitol. Each cell suspension was split into 6×70 μL aliquots, each of which was loaded into a flat-bottomed well of a 96-well black fluorescence microplate (Corning Incorporated, NY). The samples were then analysed by fluorimetry using a Biotek FLx800 microplate reader. The fluorescence of FITC-Annexin-V was quantified using excitation and emission wavelengths of 488 and 530 nm, respectively, and the fluorescence of PI was determined using emission and excitation wavelengths of 488 and 590 nm, respectively, after 30 s of gentle shaking. The

mean intrinsic background fluorescence of unstained cells was subtracted from the FITC-Annexin-V and PI fluorescence readings of double-stained cells.

Fluorimetric detection of $O_2^{\cdot-}$ in whole yeast cells

The detection of $O_2^{\cdot-}$ in whole yeast cells grown in YPE medium, in the presence or absence of 15 mM aspirin, was carried out essentially according to Szeto *et al.* (2007) with some modifications. The oxidant-sensitive fluorogenic probes MitoSOX Red and dihydroethidium (Invitrogen Molecular Probes, Oregon) were used. MitoSOX Red is selectively oxidized by mitochondrial $O_2^{\cdot-}$, forming a product that gives a red fluorescence (Piacenza *et al.*, 2007). Dihydroethidium is mostly sensitive to oxidation by cytosolic $O_2^{\cdot-}$, forming a product that fluoresces red upon binding nucleic acids (Bindokas *et al.*, 1996).

After the required period of growth in YPE medium with or without 15 mM aspirin, three aliquots each containing 4×10^7 cells were harvested. The cells were pelleted by centrifugation (3000 ***g***, 5 min) at room temperature to yield three, identically sized, yeast cell pellets. The pellets were each washed twice with 4 mL phosphate-buffered saline (PBS). One cell pellet was resuspended in 4 mL PBS, pH 7.4 and set aside as an unstained cell suspension (blank) for the determination of intrinsic cell fluorescence. The second cell pellet was resuspended in 4 mL of 5 μM MitoSOX Red working solution (10 μL of 5 mM MitoSOX Red fresh stock solution in DMSO, diluted in 10 mL of PBS), and the third pellet was resuspended in 4 mL of 5 μM dihydroethidium working solution (10 μL of 5 mM dihydroethidium stock solution in DMSO, diluted in 10 mL of PBS). Each cell suspension, including the unstained cell suspension, was then incubated for 45 min with gentle rotation in darkness at 28 °C. After incubation, the stained and unstained cell suspensions were pelleted by centrifugation (3000 ***g***, 5 min) at room temperature, and the pellets were each rapidly washed twice with 4 mL PBS. Each pellet was resuspended in 400 μL of PBS from which 6×60 μL aliquots were taken, each of which was loaded into a flat-bottomed well of a 96-well black fluorescence microplate, kept in the dark.

The samples were then analysed by fluorimetry in a Biotek FLx800 microplate reader after 30 s of shaking at low intensity. To quantify the relative fluorescence of oxidized MitoSOX Red for the detection of mitochondrial $O_2^{\cdot-}$, excitation and emission wavelengths of 510 and 580 nm, respectively, were used. Quantification of fluorescence of oxidized dihydroethidium, for the detection of cytosolic $O_2^{\cdot-}$, required excitation and emission wavelengths of 488 and 585 nm, respectively. The mean intrinsic background fluorescence signal of unstained cells was also determined and subtracted from the fluorescence readings of cells stained with MitoSOX Red or dihydroethidium.

Relative fluorescence was expressed as a percentage, obtained as the mean of measured blank-subtracted relative fluorescence units (rfu) of cells grown in the absence or presence of aspirin, normalized in each case by the mean value of the blank-subtracted rfu of cells grown in the absence of aspirin taken as 100%.

To confirm that MitoSOX Red and dihydroethidium fluorescence signals specifically indicate the accumulation of $O_2^{\cdot-}$ in the yeast cell mitochondrial matrix and cytosol, respectively, the fluorescence of both ROS probes was examined in wild-type EG103 and MnSOD-deficient EG110 yeast cells cultivated for 24 h in YPE medium in the absence or presence of 10 μM paraquat.

Separation of yeast mitochondria from cytosol and immunoscreening

The protocol used in the isolation of mitochondria and membrane-free cytosolic fraction was according to Glick & Pon (1995). Electroblotting of proteins from SDS polyacrylamide gels on to nitrocellulose membranes (Hybond-C extra, from Amersham Biosciences, UK) was carried out on a Pharmacia LKB 2117-250 Novablot electrophoresis transfer kit. For the ensuing immunoscreening of the proteins, the ECL Western Blotting Analysis System RPN 2108 (Amersham Biosciences, UK) was used.

Analysis of protein concentration and SOD activity

The protein concentration of mitochondrial and cytosolic extracts was measured using the Pierce BCA Protein Assay. Protein expression studies were carried out using SDS-PAGE followed by staining with Gelcode Blue stain reagent. The Pierce protein assay kit and Gelcode Blue stain reagent were from Pierce Biotechnology Inc., PA.

SOD activity was determined according to McCord & Fridovich (1969) and calculated basically according to Ysebaert-Vanneste & Vanneste (1980), as described below in 'Data analysis'. For SOD activity staining, using activity-polyacrylamide gels, the method of Beauchamp & Fridovich (1971) was used. Immersion of activity gels in NBT solution containing *c.* 15 mM potassium cyanide (KCN) for 30 min was also carried out to distinguish CuZnSOD bands from MnSOD and FeSOD bands as KCN selectively inhibits the activity of CuZnSOD (Asada *et al.*, 1974). Moreover, to detect the presence of FeSOD, activity gels were immersed in a solution of 5 mM H_2O_2 for 30 min, with gentle shaking, prior to staining with

NBT. Hydrogen peroxide inactivates FeSOD and CuZnSOD but not MnSOD (Asada *et al.*, 1975).

Measurement of mitochondrial NAD(P)H in whole yeast cells

The *in vivo* measurement of mitochondrial NAD(P)H was mainly adapted from Nieminen *et al.* (1997). It was based on the fact that near-UV excited autofluorescence of living cells originates from mitochondrial NAD(P)H with negligible contribution from the cytoplasm. Furthermore, mitochondrial NAD(P)H becomes nonfluorescent when oxidized. This allows for suitable *in vivo* fluorimetric evaluation of the redox state of mitochondrial NAD(P)H (Eng *et al.*, 1989).

After the required period of growth in YPE medium in the absence or presence of 15 mM aspirin, a yeast cell suspension of 1×10^7 cells mL^{-1} was harvested. From this cell suspension, 3×1 mL aliquots were withdrawn and the cells were pelleted by centrifugation (3000 ***g***, 5 min) at room temperature into three equally sized pellets. Each pellet was washed twice with 1 mL of PBS. One cell pellet was resuspended in 1 mL of PBS and another pellet was resuspended in 1 mL of PBS containing 5 mM KCN to achieve maximum reduction of mitochondrial NAD(P)H. The third cell pellet was resuspended in 1 mL of PBS containing 10 μM carbonyl cyanide 3-chloro-phenylhydrazone (CCCP), a known uncoupler of mitochondria, and left to incubate with gentle rotation for 5 min at room temperature, in order to obtain maximum oxidation of intracellular mitochondrial NAD(P)H. Each of the three yeast cell suspensions was aliquoted into 8×100 μL volumes, which were loaded in a 96-well black fluorescence microplate. Equal volumes of blank solutions of PBS, PBS with 5 mM KCN and PBS with 10 μM CCCP were also loaded in the fluorescence microplate (100 μL per well), together with the cell samples, for background subtraction. The near-UV autofluorescence of all cell samples and blank solutions was measured in a Biotek FLx800 microplate reader after 30 s of shaking at low intensity, using excitation and emission wavelengths of 350 and 420 nm, respectively. The mean background autofluorescence signals were subtracted from the autofluorescence signals of the cell samples contained in PBS (F_1), PBS with 5 mM KCN (F_2) and PBS with 10 μM CCCP (F_3), respectively. Using the corrected autofluorescence signal readings, the mitochondrial NAD(P)H (%) in the yeast cell samples was then calculated as $(F_1-F_3)/(F_2-F_3) \times 100$.

Data analysis

To calculate SOD activity, the ratio of blank to sample reaction rate (V_b/V_s) was plotted against the *Assay Sample Volume* × *Protein Concentration/Dilution of Test Sample*. In this case, the regression coefficient (or slope) is the SOD activity of the test sample given directly in U mg^{-1} protein. The plots were fitted by biweight regression (Mosteller & Tukey, 1977). EG110F cytosolic SOD activity was partitioned into CuZnSOD activity and FeSOD activity according to Ysebaert-Vanneste & Vanneste (1980) using a dissociation constant of 0.14 mM at pH 7.8 for the CuZnSOD-KCN complex calculated from data for KCN inhibition of EG103 cytosolic SOD activity. The SE was estimated by propagation of variance in the calculation. The statistical significance of changes seen with aspirin was obtained by means of a Welch-type *t*-test (Zar, 2010). Otherwise, statistical significance of the difference between total SOD activities, obtained as regression coefficients, was determined by the two-tailed *t*-test with checking for inequality of residual variance for the regressions (Bailey, 1959).

The statistical significance of differences between mean observations without and with paraquat was also determined by means of a Welch-type *t*-test (Zar, 2010). For other data, the statistical significance of differences between mean observations without and with aspirin was determined by means of the unpaired two-tailed *t*-test with checking for inequality of variance (Bailey, 1959). The nonparametric Mann–Whitney *U*-test (Zar, 2010) was used to support the results of the *t*-tests.

Results

Escherichia coli FeSOD targeted to the mitochondria of *S. cerevisiae* EG110 cells deficient in MnSOD rescues the aspirin-induced apoptotic phenotype

In our previous work (Balzan *et al.*, 2004), we observed that 15 mM aspirin committed MnSOD-deficient EG110 yeast cells growing in YPE medium to apoptotic cell death. To ascertain that it was the absence of mitochondrial SOD that was responsible for aspirin-induced apoptosis, we targeted *E. coli* FeSOD to the mitochondria of MnSOD-deficient *S. cerevisiae* EG110 cells. The yeast plasmid vectors YEp/PGK and YEp/PGK-S (YEp/PGK containing the *E. coli* FeSOD gene with the yeast MnSOD gene leader sequence) used for the transformation of *S. cerevisiae* EG110 cells gave rise to the recombinant strains EG110YEp (used as control) and EG110F, respectively. Fluorimetric detection of apoptosis, using FITC-Annexin-V and PI double-staining, was carried out on *S. cerevisiae* EG103 (wild-type), EG110YEp (MnSOD-deficient) and EG110F (expressing *E. coli* FeSOD in the mitochondria) yeast cells growing in YPE medium, in the absence and presence of 15 mM aspirin. A significant

increase in the fluorescence of FITC-Annexin-V but not of PI indicates an increase in apoptotic cells, whilst a significant increase in both FITC-Annexin-V and PI fluorescence indicates an increase in necrotic cells. Unchanged fluorescence of both FITC-Annexin-V and PI indicates the absence of an apoptotic or necrotic phenotype with respect to the control cell population growing in YPE medium in the absence of aspirin. From the results, it was clear that, whereas EG110YEp cells growing in YPE medium in the presence of aspirin were committed to apoptotic cell death (Fig. 1a), this did not happen in EG110F (Fig. 1b) and EG103 (Fig. 1c) cells, where the presence of mitochondrial SOD in both strains prevented the induction of apoptosis by aspirin in ethanol medium.

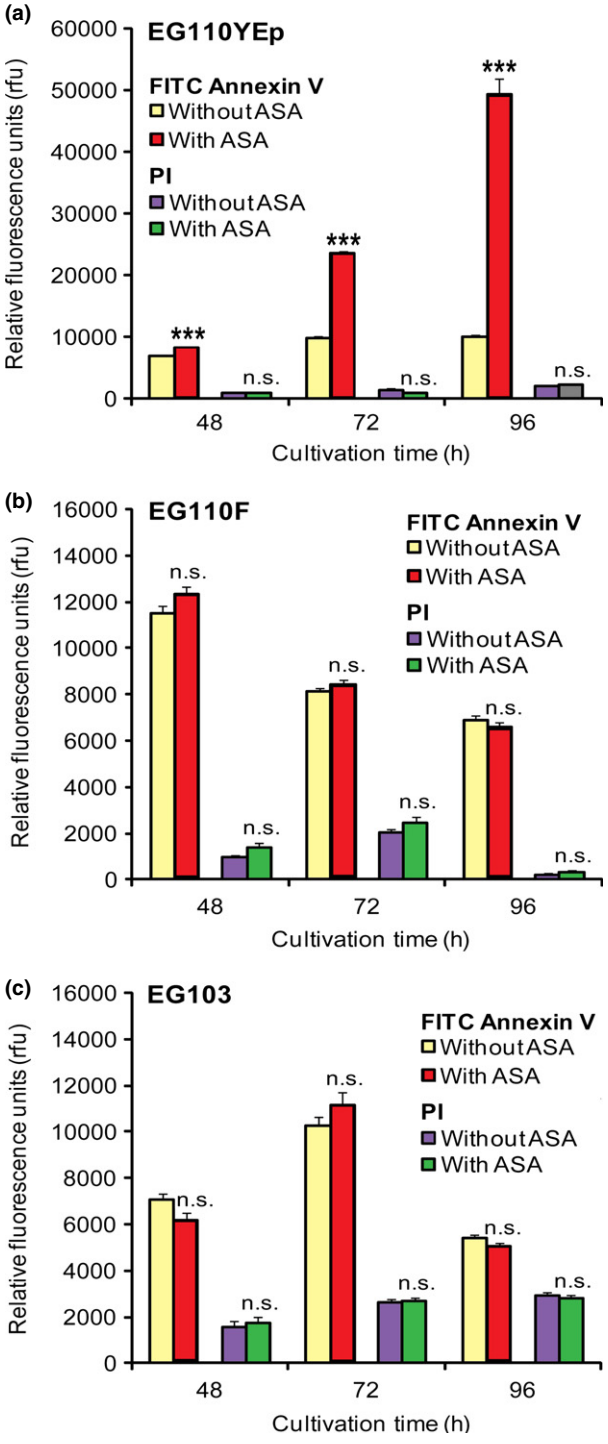

Fig. 1. Aspirin (ASA)-induced apoptosis in MnSOD-deficient yeast cells growing in ethanol medium. Screening for apoptosis in *Saccharomyces cerevisiae* (a) EG110YEp (MnSOD-deficient), (b) EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and (c) EG103 (wild-type) yeast cells grown aerobically in YPE medium was carried out in the absence and presence of 15 mM aspirin. The rfu of both FITC-Annexin-V and PI stains in whole yeast cells was measured. FITC-Annexin-V fluorescence readings in cells without and with aspirin are represented by yellow and red bars, respectively. PI fluorescence readings of cells without and with aspirin are represented by purple and green bars, respectively. A highly significant increase in the fluorescence of FITC-Annexin-V but not of PI indicates an increase in apoptotic cells, whilst a highly significant increase in both FITC-Annexin-V and PI fluorescence indicates an increase in necrotic cells. Unchanged fluorescence of both FITC-Annexin-V and PI indicates the absence of any increased apoptotic or necrotic phenotype with respect to the control cell population without aspirin. Background fluorescence of unstained cells was subtracted from the data. Vertical bars represent means and error bars, which appear where sufficiently large, represent the SE based on an average of 18 experimental points for the incubation times in the various panels. ns, not significant ($P > 0.05$); ***, highly significant ($P < 0.001$); *t*-test. (For EG103 FITC-Annexin-V fluorescence at 48 h, the *t*-test indicated $P = 0.0591$ and the Mann–Whitney *U*-test indicated $P = 0.0235$).

The effect of aspirin on levels of mitochondrial and cytosolic $O_2^{\cdot-}$ in yeast cells growing in YPE medium

Quantification of the relative fluorescence of oxidized MitoSOX Red for the detection of mitochondrial $O_2^{\cdot-}$, and oxidized dihydroethidium for the detection of cytosolic $O_2^{\cdot-}$, was carried out as described in 'Materials and methods'. The specificity of MitoSOX Red and dihydroethidium for mitochondrial and cytosolic $O_2^{\cdot-}$, respectively, was confirmed using paraquat. Paraquat induces mitochondrial accumulation of $O_2^{\cdot-}$ by redox-cycling, causing extensive mitochondrial oxidative damage, as has been observed in both yeast and mammalian cells (Cochemé & Murphy, 2008). In *S. cerevisiae* EG110 cells, which lack mitochondrial MnSOD but have cytosolic CuZnSOD, paraquat induced a highly significant increase in MitoSOX Red fluorescence, but no significant increase in dihydroethidium fluorescence (Fig. 2a). On the other

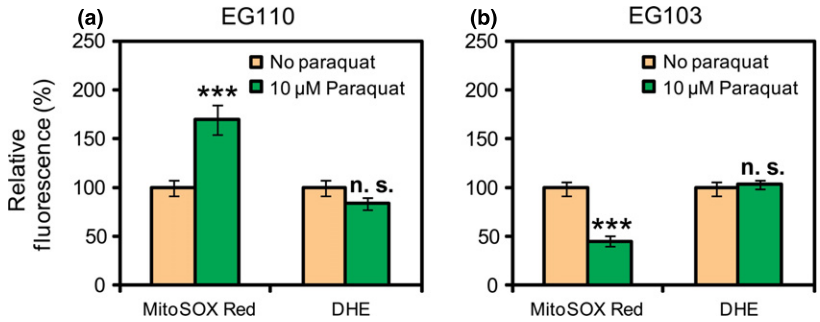

Fig. 2. Effect of paraquat on intracellular levels of superoxide radicals ($O_2^{\cdot-}$) of yeast cells growing in ethanol medium. Normalized relative fluorescence levels (%) of mitochondrial and cytosolic $O_2^{\cdot-}$ are shown in *Saccharomyces cerevisiae* (a) EG110 (MnSOD-deficient) and (b) EG103 (wild-type) cells, grown aerobically for 24 h in YPE medium in the absence (orange bars) and presence (green bars) of 10 μM paraquat. Whole yeast cells were treated with the fluorogenic probes MitoSOX Red and dihydroethidium for the direct detection and quantification of mitochondrial and cytosolic $O_2^{\cdot-}$, respectively. Background fluorescence was subtracted, and the data with paraquat were normalized with respect to those without paraquat. Vertical bars represent means, and error bars, which appear where sufficiently large, represent the SE based on data from three separate experiments in each case. ns, not significant ($P > 0.05$); ***, highly significant ($P < 0.001$); *t*-test.

hand, in EG103 cells, which have both mitochondrial MnSOD and cytosolic CuZnSOD, paraquat induced a highly significant decrease in MitoSOX Red fluorescence and no significant change in dihydroethidium fluorescence (Fig. 2b). These results show that it is oxidized MitoSOX Red and not dihydroethidium that specifically fluoresces in response to mitochondrial $O_2^{\cdot-}$ accumulation in EG110 cells. Dihydroethidium, a probe known to be sensitive to cytosolic $O_2^{\cdot-}$ accumulation in yeast (Solans *et al.*, 2006; Quaranta *et al.*, 2011; Rinnerthaler *et al.*, 2012) and mammalian cells (Gao & Wolin, 2008; Sakellariou *et al.*, 2013), did not show a significant change in fluorescence in the presence of paraquat in EG110 and EG103 cells, presumably because of the presence of cytosolic CuZnSOD in both strains.

Our next step was to investigate any potential aspirin-induced changes in $O_2^{\cdot-}$ levels in *S. cerevisiae* EG110YEp, EG110F and EG103 cells growing in YPE medium. A significant increase in the relative fluorescence (%) of oxidized MitoSOX Red and of oxidized dihydroethidium was observed in EG110YEp cells in the presence of aspirin, at 48, 72 and 96 h of aerobic growth in YPE medium (Fig. 3a and d). This indicated that EG110YEp cells experienced a significant and sustained accumulation of both mitochondrial and cytosolic $O_2^{\cdot-}$ in the presence of aspirin.

In the recombinant EG110F yeast cells (expressing *E. coli* FeSOD in the mitochondria), there was a significant increase in the mitochondrial $O_2^{\cdot-}$ levels at 48 h of growth, whereas at 72 and 96 h, there was a significant decrease in mitochondrial $O_2^{\cdot-}$ in the presence of aspirin, as indicated by the relative fluorescence (%) of oxidized MitoSOX Red (Fig. 3b). On the other hand, a significant decrease in cytosolic $O_2^{\cdot-}$ in aspirin-treated EG110F yeast cells was observed at all levels of growth studied, as indicated by the relative fluorescence (%) of oxidized dihydroethidium (Fig. 3e).

In EG103 wild-type yeast cells (with mitochondrial MnSOD), a significant decrease in mitochondrial $O_2^{\cdot-}$ was observed at 48, 72 and 96 h of growth in YPE medium, in the presence of aspirin as indicated by the relative fluorescence (%) of oxidized MitoSOX Red (Fig. 3c). The relative fluorescence (%) of oxidized dihydroethidium (Fig. 3f) indicated a significant decrease in cytosolic $O_2^{\cdot-}$ in the presence of aspirin at 48 and 72 h of growth. However, at 96 h, cytosolic $O_2^{\cdot-}$ increased significantly in the presence of aspirin. This did not cause an apoptotic phenotype in EG103 yeast cells (Fig. 1c).

Collectively, these results indicate that both the surviving yeast strains (EG103 and EG110F) managed to control and actually reduce the quantity of mitochondrial $O_2^{\cdot-}$ in the presence of aspirin. Even though EG103 yeast cells experienced a pronounced accumulation of cytosolic $O_2^{\cdot-}$ at 96 h of growth, the cells survived in YPE medium in the presence of aspirin. This indicates that the accumulation of cytosolic $O_2^{\cdot-}$ was not critically responsible for the aspirin-induced apoptotic phenotype in *S. cerevisiae* cells deficient in mitochondrial SOD. In fact, the MnSOD-deficient EG110YEp yeast strain, the only one in this study to undergo aspirin-induced apoptosis, was also the only strain to experience an accumulation of mitochondrial $O_2^{\cdot-}$ at all the specified hours of growth. The apoptotic phenotype in this strain was rescued by *E. coli* FeSOD targeted to the mitochondria. It can therefore be concluded that the sustained accumulation of mitochondrial $O_2^{\cdot-}$ was the critical cause of aspirin-induced apoptosis in MnSOD-deficient yeast cells, in YPE medium.

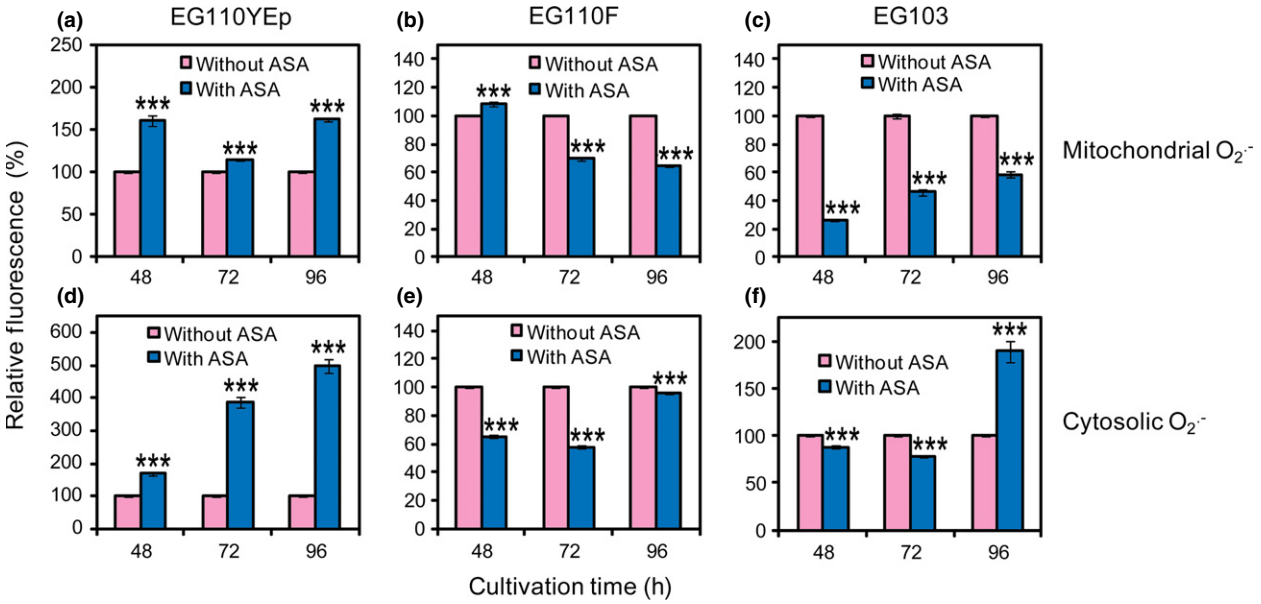

Fig. 3. Effect of aspirin (ASA) on intracellular levels of superoxide radicals ($O_2^{\cdot-}$) of yeast cells growing in ethanol medium. Normalized relative fluorescence levels (%) of mitochondrial $O_2^{\cdot-}$ (a–c) and cytosolic $O_2^{\cdot-}$ (d–f) are shown, respectively, from left to right, in *Saccharomyces cerevisiae* EG110YEp (MnSOD-deficient); EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and EG103 (wild-type) cells, grown aerobically in YPE medium in the absence (pink bars) and presence (blue bars) of 15 mM aspirin. Whole yeast cells were treated with the fluorogenic probes MitoSOX Red and dihydroethidium for the direct detection and quantification of mitochondrial and cytosolic $O_2^{\cdot-}$, respectively. Background fluorescence was subtracted, and the data with aspirin were normalized with respect to those without aspirin at each incubation time. Vertical bars represent means and error bars, which appear where sufficiently large, represent the SE based on an average of 14 experimental points for the incubation times in the various panels. ***, highly significant ($P < 0.001$); *t*-test. (For EG110F cellular $O_2^{\cdot-}$ at 96 h, the *t*-test indicated $P = 0.0006$ and the Mann–Whitney *U*-test indicated $P = 0.0011$).

Aspirin-induced changes of mitochondrial and cytosolic SOD activity in yeast cells growing in YPE medium

Additionally, we investigated the protective role of mitochondrial SOD against the adverse effects of aspirin by studying any possible aspirin-induced changes of yeast mitochondrial and cytosolic SOD activity in *S. cerevisiae* EG110YEp (MnSOD-deficient), EG110F (expressing *E. coli* FeSOD in the mitochondria) and EG103 (wild-type) yeast cells growing in YPE medium, in the absence and presence of 15 mM aspirin. Spectrophotometric assay of the levels of SOD activity was carried out on the mitochondrial and cytosolic extracts of the yeast cells at 48, 72 and 96 h of growth. In parallel to the SOD activity assays, polyacrylamide gels, together with SOD activity staining, were also used in comparative studies of the mitochondrial and cytosolic SOD activities of the yeast cells, in the presence and absence of aspirin, as cited in 'Materials and methods'.

We confirmed that there was no cross-contamination between any of the mitochondrial and cytosolic protein extracts of the yeast cells, by carrying out immunoscreening of the mitochondrial and cytosolic extracts of EG110YEp (Fig. 6a), EG110F (Fig. 6b) and EG103 (Fig. 6c) yeast cells using monoclonal *anti*-β-actin and polyclonal *anti*-Hsp60 as primary antibodies.

In the MnSOD-deficient yeast strain EG110YEp, there was a significant increase in cytosolic CuZnSOD activity in the presence of aspirin, at all levels of growth (Fig. 4b). This was corroborated by the activity gel results shown in Fig. 5b, where the achromatic band of active cytosolic SOD, proved to be CuZnSOD on inhibition by KCN, was noticeably more intense in the presence of aspirin, at 48, 72 and 96 h of growth. However, the sustained increase in CuZnSOD activity did not prevent $O_2^{\cdot-}$ accumulation (Fig. 3a and d) and apoptosis (Fig. 1a) of EG110YEp yeast cells, during cultivation in YPE medium in the presence of aspirin.

A trace amount of mitochondrial CuZnSOD activity was also observed in EG110YEp cells (Figs 4a and 5a) which, as shown by the immunoscreening results in Fig. 6a, was not due to contamination from the cytosolic extracts. This mitochondrial CuZnSOD did not change significantly in response to aspirin treatment, neither did it prevent $O_2^{\cdot-}$ accumulation (Fig 3a and d) and apoptosis (Fig. 1a) of EG110YEp yeast cells during cultivation in YPE medium in the presence of aspirin.

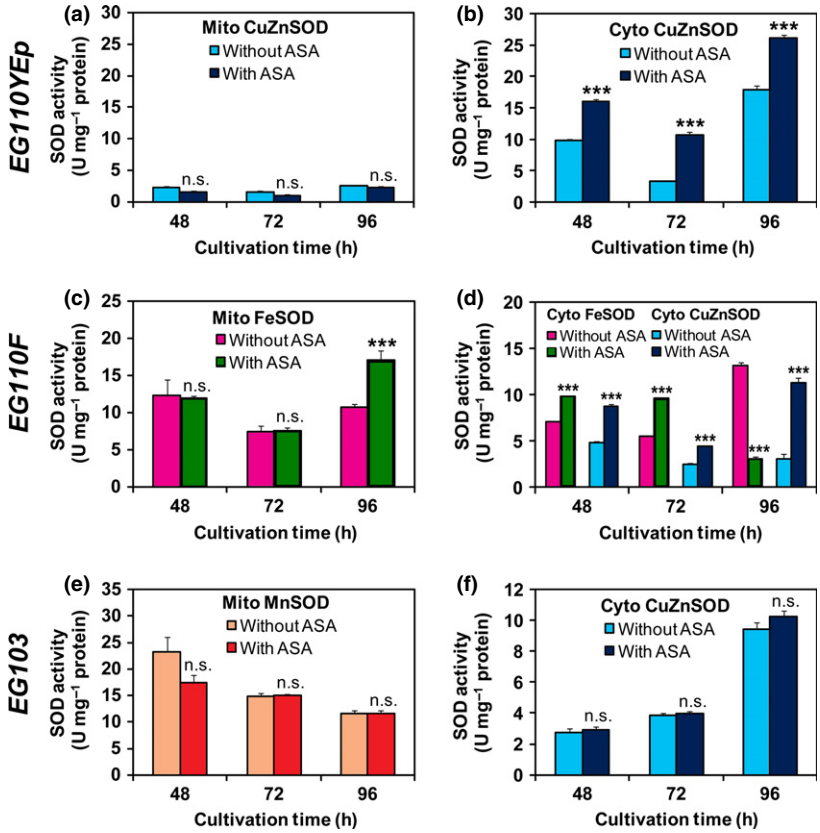

Fig. 4. SOD activity levels in the absence and presence of aspirin (ASA). (a), (c) and (e) show SOD activity levels (U mg^{-1} of protein) in mitochondrial extracts, and (b), (d) and (f) show SOD activity levels (U $mg^{-1}$of protein) in cytosolic extracts of *Saccharomyces cerevisiae* EG110YEp (MnSOD-deficient), EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and EG103 (wild-type) cells grown aerobically in YPE medium in the absence (magenta bars for FeSOD, orange bars for MnSOD and light blue bars for CuZnSOD) and presence of 15 mM aspirin (green bars for FeSOD, red for MnSOD and dark blue for CuZnSOD). Activity attributable to intermembrane space CuZnSOD in EG110F or EG103 mitochondrial SOD determinations was about 5% of total. SOD activity was measured according to McCord & Fridovich (1969) and basically calculated according to Ysebaert-Vanneste & Vanneste (1980) as described in 'Materials and methods'. Vertical bars represent the calculated SOD activity and error bars, which appear where sufficiently large, represent the SE based on an average of 14 experimental points for the incubation times in the various panels. ns, not significant ($P > 0.05$); ***, highly significant ($P < 0.001$); *t*-test.

In the recombinant EG110F yeast strain, cytosolic Cu-ZnSOD activity increased significantly in the presence of aspirin at 48, 72 and 96 h of growth in YPE medium (Figs 4d and 5d). Cytosolic FeSOD activity also showed a significant increase at 48 and 72 h of growth, in the presence of aspirin. However, at 96 h, aspirin induced a significant decrease in cytosolic FeSOD activity (Figs 4d and 5d), at which point it also induced a significant increase in mitochondrial FeSOD activity (Figs 4c and 5c). Hence, in EG110F recombinant yeast cells, aspirin initially caused a significant increase in cytosolic SOD activity, followed by a significant increase in mitochondrial SOD activity at 96 h of growth.

In the wild-type EG103 cells, no significant aspirin-induced changes in mitochondrial MnSOD activity (Figs 4e and 5e) or cytosolic CuZnSOD activity (Figs 4f and 5f) were observed at 48, 72 and 96 h of growth in YPE medium, and the levels of mitochondrial $O_2^{\cdot-}$ were reduced in the presence of aspirin at all stages of growth (Fig. 3c). This was in contrast to what happened in EG110F cells where, besides an aspirin-induced increase in cytosolic CuZnSOD (Figs 4d and 5d), an increase in the cloned mitochondrial FeSOD (Figs 4c and 5c) was required to maintain a significant reduction of mitochondrial $O_2^{\cdot-}$ (Fig. 3b).

Aspirin-induced oxidation of mitochondrial NAD(P)H in MnSOD-deficient yeast cells growing in YPE medium

A study was carried out to determine whether aspirin-induced apoptosis of MnSOD-deficient yeast cells, in which there was an accumulation of mitochondrial $O_2^{\cdot-}$ (Fig. 3a), was associated with the oxidation of mitochondrial NAD

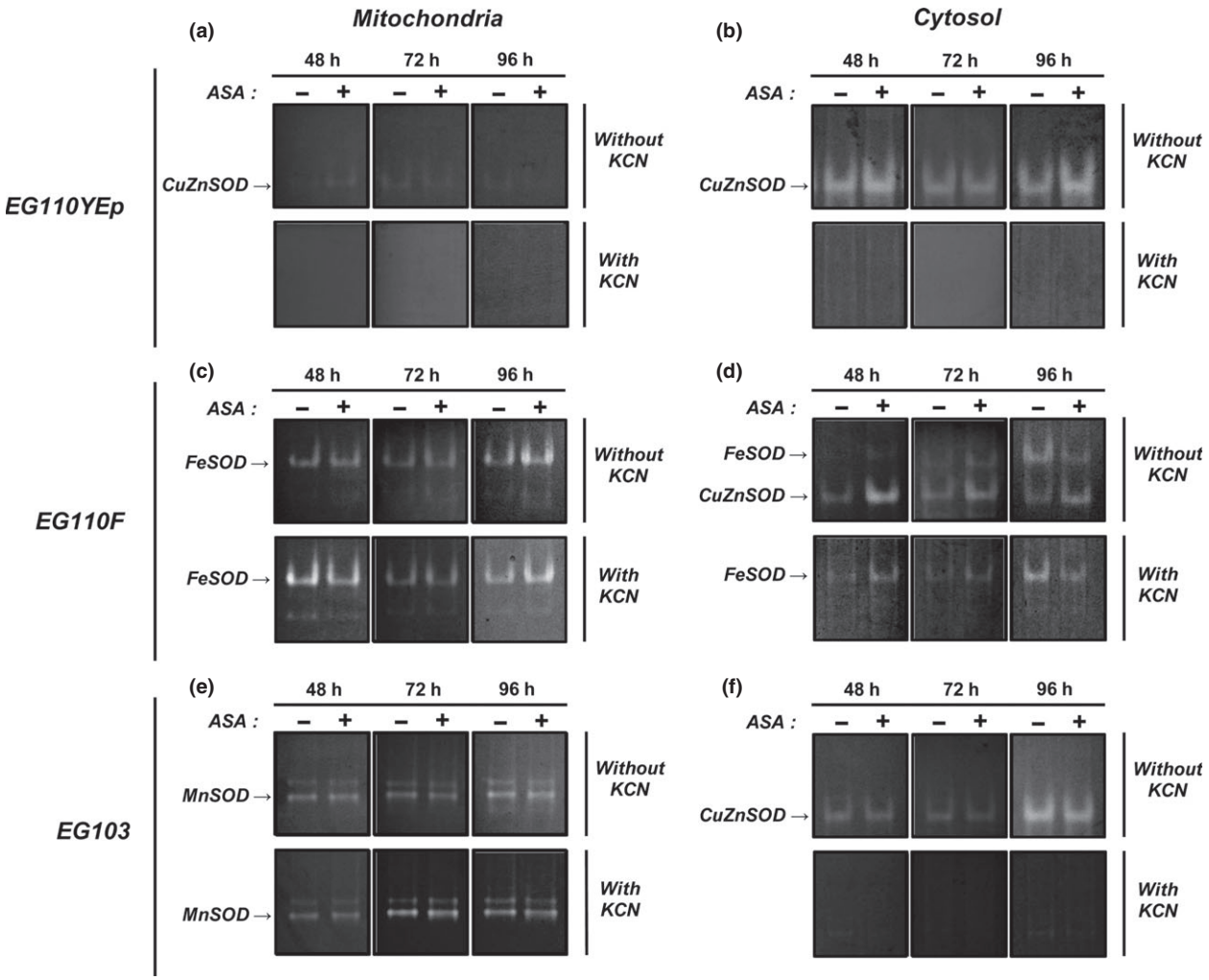

Fig. 5. SOD polyacrylamide gel activity stains in the absence and presence of aspirin (ASA). (a), (c) and (e) show SOD gel activity stains of mitochondrial extracts, and (b), (d) and (f) show SOD gel activity stains of cytosolic extracts of *Saccharomyces cerevisiae* EG110YEp (MnSOD-deficient), EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and EG103 (wild-type) cells grown aerobically in YPE medium in the absence and presence of 15 mM aspirin. Equal amounts of mitochondrial and cytosolic protein extracts were loaded in their respective wells and passed through 5% (w/v) stacking, 10% (w/v) resolving polyacrylamide native gels followed by SOD activity staining with 2.45 mM nitroblue tetrazolium. For each cultivation time, the lower panels show activity stains of gels treated with 2.45 mM nitroblue tetrazolium containing 15 mM KCN to inactivate CuZnSOD.

(P)H. Comparative measurements of the level of mitochondrial NAD(P)H (%) in *S. cerevisiae* EG110YEp (MnSOD-deficient), EG110F (expressing *E. coli* FeSOD in the mitochondria) and EG103 (wild-type) yeast cells growing in YPE medium, in the absence or presence of aspirin, were done at 48, 72 and 96 h of growth. Near-UV autofluorescence of whole yeast cells, which decreases when mitochondrial NAD(P)H is oxidized, was quantified as described in 'Materials and methods'. This was repeated for each strain after treatment with 5 mM KCN (which causes maximum reduction of the mitochondrial NAD(P)H) and 10 μM CCCP (which causes maximum oxidation of the mitochondrial NAD(P)H).

As shown in Fig. 7a, there was a significant decrease in the percentage level of mitochondrial NAD(P)H in MnSOD-deficient EG110YEp cells, at all levels of growth, in the presence of aspirin. This is a clear indication that aspirin caused significant oxidation of mitochondrial NAD(P)H in EG110YEp cells growing in YPE medium. Aspirin also caused a significant decrease in the percentage level of mitochondrial NAD(P)H in EG110F yeast cells at 48 h of growth in YPE medium (Fig. 7b). However, at 72 and 96 h of growth, there was a significant increase in the level of mitochondrial NAD(P)H, in the presence of aspirin. Hence, the EG110F cells, which express *E. coli* FeSOD in the mitochondria, managed to

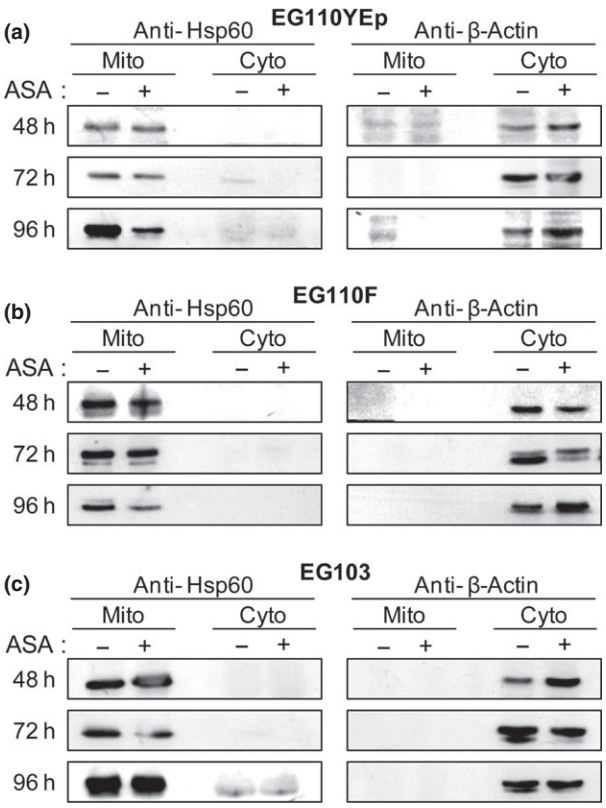

Fig. 6. Immunoscreening by chemiluminescence to confirm lack of contamination of mitochondrial extract by cytosolic extract and vice versa. Immunoscreening by chemiluminescence for the mitochondrial matrix protein Hsp60 and the cytosolic protein β-actin in both the mitochondrial and cytosolic extracts of *Saccharomyces cerevisiae* (a) EG110YEp (MnSOD-deficient), (b) EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and (c) EG103 (wild-type) cells grown aerobically in YPE medium in the absence (–) and presence (+) of aspirin (ASA). The electroblotting on to the nitrocellulose membranes was carried out from 5% (w/v) stacking, 12% (w/v) resolving acrylamide gels after SDS-PAGE as described in 'Materials and methods'. The two left lanes and the two right lanes of each immunoblot were loaded with equal amounts of mitochondrial and cytosolic extracts, respectively.

suppress the oxidative effect of aspirin and effectively preserve the percentage level of mitochondrial NAD(P)H during later growth. In EG103 wild-type yeast cells, aspirin did not induce any significant change in the percentage level of mitochondrial NAD(P)H at 48 h of growth in YPE medium (Fig. 7c). Furthermore, as was observed in EG110F cells (Fig. 7b), EG103 cells managed not only to suppress aspirin-mediated oxidation of mitochondrial NAD(P)H, but also to increase the percentage level of mitochondrial NAD(P)H, at 72 and 96 h of growth in YPE medium, in the presence of aspirin (Fig. 7c). These results strongly suggest that the sustained increase in mitochondrial $O_2^{\cdot-}$, in the absence of mitochondrial SOD, was essentially responsible for the aspirin-induced oxidation of mitochondrial NAD(P)H in *S. cerevisiae* cells growing in ethanol medium.

Discussion

In previous studies, we established the role of mitochondrial MnSOD as a critical defence against aspirin-induced apoptosis in yeast cells grown in nonfermentable ethanol medium (Balzan *et al.*, 2004). Given the fact that SOD eliminates $O_2^{\cdot-}$ (McCord & Fridovich, 1969), there was a strong indication that aspirin-induced toxicity in MnSOD-deficient yeast cells growing in YPE medium was mediated by the accumulation of $O_2^{\cdot-}$ in the mitochondria. However, when the oxidant-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate was used to study the intracellular oxidation levels in these cells, aspirin appeared to exert an antioxidant effect until the onset of late apoptosis, when a moderately significant accumulation of ROS was observed (Balzan *et al.*, 2004). Considering that the response of SODs to the presence of aspirin in other biological systems is not well understood, it became clear that besides ROS levels, the cytoprotective role of mitochondrial SOD against aspirin required further investigation.

In this work, we confirmed the apoptotic effect of aspirin in the MnSOD-deficient *S. cerevisiae* EG110YEp cells in YPE medium (Fig. 1a). In contrast, the targeting of *E. coli* FeSOD to the mitochondria of MnSOD-deficient yeast cells rescued them from the pro-apoptotic effect of aspirin and, similar to the wild-type EG103 cells (Fig. 1c), the recombinant EG110F yeast cells (with exogenous *E. coli* FeSOD targeted to the mitochondrial matrix) overcame the growth-inhibitory effects of aspirin (Fig. 1b). In recombinant MnSOD-deficient *S. cerevisiae* DL1$Mn^{-}Fe^{+}$-P cells, Balzan *et al.* (1995) observed that the yeast MnSOD targeting signal was not cleaved off from the FeSOD protein after entry into the mitochondrial matrix. Furthermore, this did not affect the activity of the SOD enzyme, as observed also in EG110F cells (Figs 4c and 5c) in the present work. In fact, the presence of some active FeSOD was also observed in the cytosolic extracts of EG110F yeast cells (Figs 4d and 5d) which were free of any mitochondrial contaminant proteins (Fig. 6b). It is unlikely that the cytosolic FeSOD in recombinant EG110F cells played a critical cytoprotective role against the pro-apoptotic effects of aspirin. In aspirin-treated EG110F cells grown for 96 h in YPE medium, the cytosolic FeSOD activity showed a significant decrease (Figs 4d and 5d), and yet these cells still grew efficiently and did not undergo apoptosis (Fig. 1b). It was the aspirin induced increase in mitochondrial FeSOD at 96 h (Figs 4c and 5c) which seemed to have saved the EG110F yeast cells from apoptosis. In fact, the continuous aspirin-induced increase in cytosolic

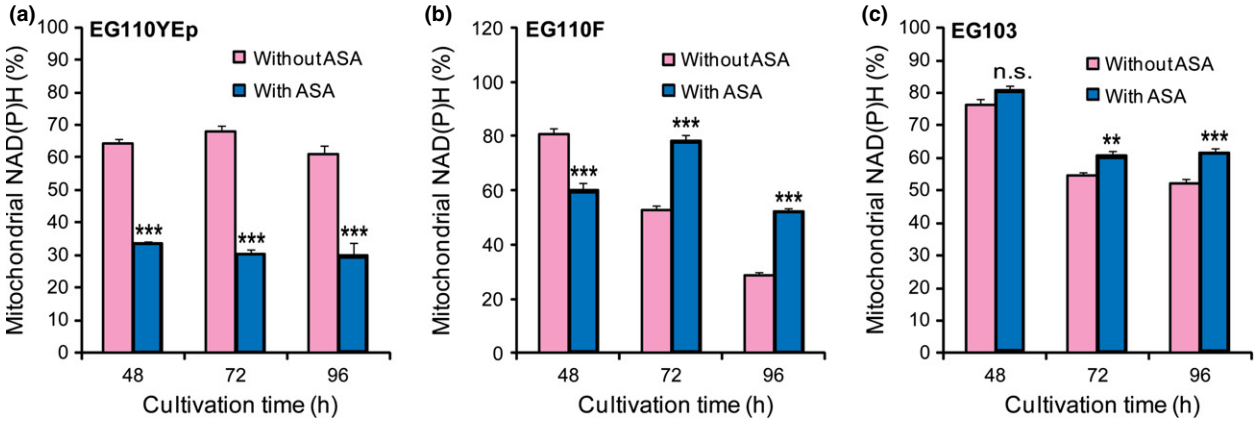

Fig. 7. Effect of aspirin (ASA) on mitochondrial NAD(P)H of yeast cells growing in ethanol medium. Levels of reduced mitochondrial NAD(P)H were measured in *Saccharomyces cerevisiae* (a) EG110YEp (MnSOD-deficient), (b) EG110F (expressing *Escherichia coli* FeSOD in the mitochondria) and (c) EG103 (wild-type) cells grown aerobically in YPE medium in the absence (pink bars) and presence (blue bars) of 15 mM aspirin. Near-UV autofluorescence of whole yeast cells, which decreases when mitochondrial NAD(P)H is oxidized, was quantified. This was repeated for each strain after treatment with 5 mM KCN (which causes maximum reduction of mitochondrial NAD(P)H) and 10 μM CCCP (which causes maximum oxidation of mitochondrial NAD(P)H). Levels of reduced mitochondrial NAD(P)H are shown as percentages of the range indicated by CCCP and KCN as described in 'Materials and methods'. Vertical bars represent means and error bars, which appear where sufficiently large, represent the SE based on an average of 28 experimental points for the incubation times in the various panels. ns, not significant ($P > 0.05$); **, moderately significant ($0.001 < P < 0.01$); ***, highly significant ($P < 0.001$); *t*-test.

CuZnSOD in EG110YEp cells (Fig. 4b), which was similar to the sustained increase in cytosolic CuZnSOD in EG110F cells (Fig. 4d), did not prevent the apoptotic phenotype in EG110YEp cells. This is corroborated by our previous work that showed that *S. cerevisiae* EG118 yeast cells, which have mitochondrial MnSOD but are deficient in cytosolic CuZnSOD, survived the effects of aspirin in YPE medium (Balzan *et al.*, 2004).

Having confirmed that the survival of yeast cells in the presence of aspirin in YPE medium requires the presence of SOD, but not necessarily the native MnSOD, in the mitochondrial matrix, we investigated the potential involvement of $O_2^{\cdot-}$ in aspirin-induced apoptosis of yeast cells under these growth conditions. Although aspirin-induced apoptosis accompanied by the accumulation of cellular ROS has been observed in other biological systems, such as HepG2 human hepatoma cells (Raza *et al.*, 2011), it has additionally been reported that aspirin is an extremely efficient and selective scavenger of $OH^{\cdot}$ in biological systems, even if it is a poor scavenger of $O_2^{\cdot-}$ and H_2O_2 (Sagone & Husney, 1987). It has been shown that aspirin inhibits mitochondrial respiration in MnSOD-deficient yeast cells grown in YPE medium (Sapienza *et al.*, 2008), in HepG2 cells (Raza *et al.*, 2011) and in isolated mitochondria of *Nicotiana tabacum* plant cells (Norman *et al.*, 2004). Inhibition of mitochondrial electron transport is known to trigger the generation of ROS, supposedly due to the reduced respiratory consumption of oxygen, which is subsequently made more available for the generation of intracellular ROS (Perrone *et al.*, 2008). This could be the reason why, on using the fluorescent probes MitoSOX Red and dihydroethidium, which detect changes in mitochondrial and cytosolic $O_2^{\cdot-}$, respectively (Gao & Wolin, 2008; Sakellariou *et al.*, 2013), we observed that aspirin increases significantly the levels of mitochondrial and cytosolic $O_2^{\cdot-}$ in EG110YEp cells (Fig 3a and d, respectively).

It has been proposed that salicylate, the primary metabolite of aspirin, inhibits respiration and causes ROS generation in rat liver mitochondria by interacting with Complex I of the respiratory chain (Battaglia *et al.*, 2005). It is not unlikely that aspirin, which is converted to salicylate, mediates inhibition of electron transport in yeast cells by interaction of the salicylate with some component of the yeast equivalent of mitochondrial Complex I. This may conceivably promote one-electron reduction of molecular oxygen and lethal generation of $O_2^{\cdot-}$. In previous work, we observed that salicylate caused the same growth-inhibition phenotype as aspirin in MnSOD-deficient yeast cells grown in YPE medium (Sapienza & Balzan, 2005).

As explained in the results, the accumulation of mitochondrial $O_2^{\cdot-}$, which was diminished in the presence of mitochondrial SOD in both EG110F and EG103 cells (Fig 3b and c, respectively) is what probably led the MnSOD-deficient EG110YEp cells to their apoptotic cell death (Fig. 1a), given that ROS accumulation has long been implicated in apoptotic cell death in yeast (Madeo *et al.*, 1997, 1999; Ludovico *et al.*, 2002).

In aspirin-treated EG110F cells, the fact that oxidation of mitochondrial NAD(P)H increased during accumulation of mitochondrial $O_2^{\cdot-}$ at 48 h of growth and was then reversed (Fig. 7b) when mitochondrial $O_2^{\cdot-}$ levels were attenuated at 72 and 96 h of growth (Fig. 3b), at which latter time mitochondrial FeSOD activity had increased significantly (Figs 4c and 5c) is evidence of a strong correlation between the level of mitochondrial $O_2^{\cdot-}$ and the oxidation of mitochondrial pyridine nucleotides. Also, in the wild-type EG103 cells, which survived in the presence of aspirin, an increase in the level of mitochondrial NAD(P)H (Fig. 7c) is supported by the sustained decrease in mitochondrial $O_2^{\cdot-}$ (Fig. 3c) due to the very efficient dismutation of $O_2^{\cdot-}$ by mitochondrial MnSOD, the activity of which was not altered even by the presence of aspirin (Figs 4e and 5e). Hence, it is now clear that (a) the pro-apoptotic role of mitochondrial $O_2^{\cdot-}$ accumulation in aspirin-treated MnSOD-deficient yeast cells grown in ethanol medium is associated with the oxidation of mitochondrial NAD(P)H, and (b) the survival of yeast cells under these growth conditions depends on their ability to control the mitochondrial $O_2^{\cdot-}$ levels and to conserve the pool of mitochondrial NAD(P)H. These two prerequisites can only be achieved if SOD is present in the mitochondrial matrix.

To conclude, there is the possibility that the accumulation of mitochondrial $O_2^{\cdot-}$ in aspirin-treated EG110YEp cells growing in ethanol medium was caused by the aspirin-induced inhibition of mitochondrial respiration that was observed in previous work (Sapienza *et al.*, 2008). Inhibition of the electron transport chain is known to induce mitochondrial ROS accumulation (Perrone *et al.*, 2008). It is unlikely that the mitochondrial $O_2^{\cdot-}$ generated in the presence of aspirin directly oxidizes mitochondrial NAD(P)H in yeast cells. This is because direct oxidation of pyridine nucleotides by $O_2^{\cdot-}$ is very slow compared with direct oxidation of NAD(P)H by other ROS such as H_2O_2 (Petrat *et al.*, 2006). It is more probable that NAD(P)H oxidation is due to mitochondrial uncoupling that causes accelerated oxidation of mitochondrial pyridine nucleotides (Eng *et al.*, 1989; Vlessis, 1990; Nieminen *et al.*, 1997).

It is well known that salicylate, the primary metabolite of aspirin, is both an uncoupler and an inhibitor of mitochondrial electron transport (Norman *et al.*, 2004). Furthermore, whereas uncoupling of normal mitochondria decreases, uncoupling of inhibited mitochondria increases ROS generation that accentuates uncoupling (Brookes, 2005). In previous work, we observed a striking drop in the mitochondrial membrane potential ($\Delta\Psi_m$) of aspirin-treated MnSOD-deficient yeast cells (Sapienza *et al.*, 2008) that is associated with uncoupling among other mitochondrial apoptotic phenomena. Taken together our results suggest that in aspirin-induced apoptosis in *S. cerevisiae* cells, mitochondrial redox balance as evidenced by maintained decrease in mitochondrial NAD(P)H in our results is shifted in favour of oxidation, which is consistent with the findings of Cai & Jones (1998) in staurosporine-induced apoptosis in HT29 leukaemia cells.

Because several core cellular processes, such as apoptosis, are conserved among yeast and mammalian cells, the observations in this study may contribute to our understanding of the mechanistic behaviour of aspirin in mammalian cancer cells. Considering the fact that tumour cells experience constantly higher levels of oxidative stress with respect to normal cells, because of their increased metabolic rate (Qin *et al.*, 2013), aspirin-induced increase in mitochondrial $O_2^{\cdot-}$ may be enough to overwhelm cancer cell antioxidant defences such as MnSOD, resulting in apoptosis.

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