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Metabolic aspects of aspirin-induced apoptosis in yeast

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Abstract

We have previously shown that aspirin induces apoptosis in manganese superoxide dismutase (MnSOD)-deficient *Saccharomyces cerevisiae* cells cultivated in ethanol medium, and that it exhibits a significant antioxidant effect until the onset of overt apoptosis. We here report that glucose-6-phosphate dehydrogenase activity in these cells is not inhibited by aspirin. However, the reducing power, as measured by the NADPH/$NADP^+$ concentration ratio, is significantly lower than in wild-type cells. With aspirin, the levels of NADPH, $NADP^+$ and catalase in MnSOD-deficient cells decrease significantly after 72 h of cultivation, without significant decrease of the NADPH/$NADP^+$ ratio. This ratio is higher when the cells are grown in glycerol or acetate medium. This seems to prevent loss in viability and induction of apoptosis on treatment with aspirin. Additionally, the glutathione (GSH) level is maintained, but the level of oxidized glutathione (GSSG) increases, leading to a significant decrease in the GSH/GSSG ratio in aspirin-treated cells. This decrease in the GSH/GSSG ratio is much less in cells grown in glycerol medium, while there is an increase in the GSH/GSSG ratio of cells grown in acetate medium. Consequently, the decreased reducing power may be linked to apoptotic induction by aspirin. This occurs independently of the level of reactive oxygen species which, as shown in our previous studies, do not play a primary role in the apoptosis of cells exposed to aspirin. The protective effect of MnSOD appears to be related to the cellular reducing power.

Keywords: MnSOD-deficient yeast cells; Aspirin; Apoptosis; Cellular reducing power; NADPH/$NADP^+$ ratio; GSH/GSSG ratio

1. Introduction

Among unicellular organisms, apoptosis serves as a defense mechanism for the preservation of cell populations under adverse conditions, thus ensuring the survival of a few cells to propagate the genome [1]. It has been demonstrated that apoptosis in *Saccharomyces cerevisiae* is accompanied by typical features of mammalian apoptosis such as externalization of phosphatidylserine at the surface of the cytoplasmic membrane, membrane blebbing, chromatin condensation and margination, and DNA-cleavage [2]. Also, a yeast protein Yor197w with structural homology to mammalian caspases has been found to function as a caspase in yeast and was labelled Yeast Caspase-1 (YCA1) [3].

In a previous study we have investigated the apoptotic effect of aspirin on wild-type, manganese superoxide dismutase (MnSOD)-deficient and copper, zinc superoxide dismutase (CuZnSOD)-deficient *S. cerevisiae* cells. Our findings showed that MnSOD-deficient *S. cerevisiae* cells undergo aspirin-induced apoptosis when cultivated in ethanol medium. The intracellular oxidation level of these cells was measured and the results revealed that aspirin exerted a significant antioxidant effect until the onset of overt apoptosis, when there was a moderately significant increase in the intracellular oxidation level, thus suggesting that generation of reactive oxygen species (ROS) is a relatively late event and not the primary cause of cell death.

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Under the same conditions, the CuZnSOD-deficient and wild-type cells were viable. Additionally, aspirin had no inhibitory effect on the three strains when cultured on a non-fermentable carbon source such as glycerol or acetate [4].

These results led us to investigate the metabolic processes that cause cell death in MnSOD-deficient cells when cultivated in ethanol medium in the presence of aspirin. This involved determining the effect of aspirin on the activity of the enzymes glucose-6-phosphate dehydrogenase and catalase, as well as measuring the intracellular reducing power as given by NADPH/$NADP^+$ concentration ratio and by the GSH/GSSG concentration ratio. The two redox couples are linked and play an essential role in maintaining the cellular redox balance. The reducing nature of the NADPH/$NADP^+$ couple makes it an excellent source of electrons [5]. It is crucial in sustaining the glutathione system, since NADPH is required by glutathione reductase for its catalytic activity in reducing glutathione disulfide (GSSG) to two molecules of GSH. Apart from its central role in maintaining antioxidant defences, NADPH is essential in the biosynthesis of cellular macromolecules. The glutathione system also plays an essential role in yeast, since GSH functions in catalysis, metabolism and transport. Its functions include protection of cells against oxidants, maintaining reduced cellular disulfide bonds, acting as a cofactor for antioxidant enzymes, as well as detoxifying xenobiotics and heavy metals [6,7]. GSH is considered to be the major intracellular low-molecular-weight thiol due to its high concentration, and causes the intracellular reduction potential in yeast to be highly reducing [8]. When moderate oxidative stimuli render the reductive potential too positive, the cell initiates processes leading to apoptosis [5]. In this work, we tried to determine whether the redox state is perturbed, independently of the level of ROS.

2. Materials and methods

2.1. Yeast strains and culture conditions

The parent strain used in this study was *S. cerevisiae* EG103 (*MATα leu2-3 112 his3Δ1 trp1-289a ura3-52 GAL*$^+$) and the MnSOD-deficient strain was EG110 (EG103 *sod2::TRP1*), kindly provided by Edith Gralla of UCLA and Valeria C. Culotta of Johns Hopkins University. Cells were cultured in rich liquid media containing 1% Bacto yeast extract, 2% Bacto-peptone with 3% (v/v) ethanol (YPE), or 3% (v/v) glycerol (YEPG), or 2% (w/v) potassium acetate (YPA). For plates, 2% agar was used and incubation was at 28 °C. Aerobic growth in liquid culture was maintained at 28 °C with constant shaking at 250 rpm. Cells were also cultured in fresh media in the presence of 15 mM aspirin (acetylsalicylic acid) (Sigma, St. Louis, MD, USA). The pH of the media was adjusted to 5.5 with 1-M Trizma base (Sigma), with the exception of YPA where the pH was already 5.8. For treatment with 15 mM sodium salicylate the pH was adjusted to 5.5 with HCl. Growth was followed by monitoring the optical density at 600 nm (OD_{600}). An OD_{600} of 1.0 is equivalent to 10^7 cells ml^{-1} [9]. Cultures with OD_{600} values greater than 1.0 were diluted as necessary.

2.2. Enzyme activity measurements

Cellular protein extracts were prepared by glass bead lysis as described by Longo et al. [9]. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was determined spectrophotometrically by monitoring the increase in absorbance of NADPH at 339 nm [10]. Catalase (EC 1.11.1.6) activity was assayed by spectrophotometric measurement of the rate of decomposition of H_2O_2 at 240 nm [11]. Protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin as protein standard.

2.3. Determination of nicotinamide-adenine dinucleotide phosphate levels and glutathione content in yeast cells

The cellular levels of $NADP^+$ and NADPH were determined in neutralized acid and alkaline extracts, respectively, by the method of Klingenberg [12], and were expressed as nanomoles per A_{600} turbidimetric unit of culture as described by Ting et al. [13]. Concentrations of GSH and GSSG were determined by the method of Griffith [14] and were also expressed as nanomoles per A_{600} turbidimetric unit of culture.

3. Results

3.1. The aspirin metabolite salicylate inhibits growth in MnSOD-deficient S. cerevisiae cells in YPE medium

To determine whether the cause of cell death of MnSOD-deficient *S. cerevisiae* cells (EG110), when cultivated in YPE medium in the presence of 15 mM aspirin, was due to acetylation of fundamental enzymes in metabolic processes, we treated EG110 cells in YPE medium with 15 mM sodium salicylate instead of aspirin (Fig. 1). As with the aspirin-treated cells, cellular growth was also drastically inhibited with the aspirin metabolite salicylate. Thus, the apoptotic effect of aspirin on MnSOD-deficient yeast cells in YPE medium does not seem to involve enzyme acetylation.

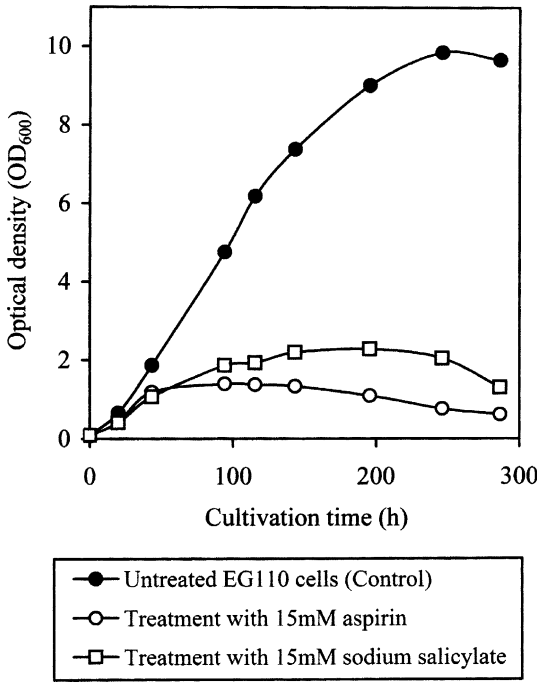

Fig. 1. Effect of 15 mM aspirin and 15 mM sodium salicylate on the growth of MnSOD-deficient *S. cerevisiae* EG110 cells cultivated in rich medium containing ethanol as the non-fermentable carbon source (YPE). The pH of the medium was adjusted to 5.5. Cell growth was estimated by measuring optical density at 600 nm. Each point represents the mean of at least three independent determinations. The SE of the means varied from 0.005 to 0.28 OD units.

3.2. Aspirin does not inactivate glucose-6-phosphate dehydrogenase but inactivates catalase in MnSOD-deficient cells in YPE medium

Next, we measured the specific activity of glucose-6-phosphate dehydrogenase in EG110 cells in YPE medium. The specific activity of the enzyme after 48 h (early exponential phase) and 72 h (mid-exponential phase) of cultivation of EG110 cells in YPE medium with 15 mM aspirin was not significantly different from that in control cells, cultivated in the absence of aspirin (Table 1). However, the specific activity of catalase in EG110 cells decreased significantly after 48 h, and after 72 h of cultivation there was more than a twofold decrease in catalase activity (Table 1). This shows that aspirin does not inhibit glucose-6-phosphate dehydrogenase activity in vivo but exerts a direct or indirect inhibitory effect on catalase activity.

3.3. Aspirin-treated MnSOD-deficient cells have reduced levels of $NADP^+$ and NADPH in YPE medium

Since aspirin was known to exert an antioxidant effect, but still commits MnSOD-deficient cells to apoptosis in ethanol medium [4], we asked whether aspirin was causing an imbalance of the redox status of the cells. NADPH is a critical modulator of the redox potential. Hence, we measured the NADPH/$NADP^+$ ratio as an indicator of the intracellular reducing power. This showed that the reducing power in EG110 cells after 72 h of cultivation in YPE medium was only 22% of that in wild-type EG103 cells (Table 2). However, EG110 cells were viable in ethanol medium in the absence of aspirin (Fig. 1). On treatment with aspirin, the levels of NADPH and $NADP^+$ in EG110 cells cultivated for 48 h in YPE medium decreased by ~30%. Moreover, after 72 h, both NADPH and $NADP^+$ decreased dramatically to one-third of the respective levels in untreated cells, without significant decrease of the NADPH/$NADP^+$ ratio (Table 2). Even though there was no change in the NADPH/$NADP^+$ ratio on treatment with aspirin, the cells were committed to eventual apoptosis. The depletion of the intracellular pyridine nucleotide level (NADPH and $NADP^+$) occurred well before typical changes associated with late apoptosis, such as phosphatidylserine exposure on the cell surface and nuclear DNA fragmentation [4].

The NADPH/$NADP^+$ ratio was higher in EG110 cells when grown in glycerol or acetate medium. Although on treatment with aspirin there was a decrease

Table 1
Enzyme activities in *S. cerevisiae* cells grown in the absence and presence of aspirin

Strains[a]	Cultivation time (h)[b]	Specific activity (unit mg^{-1} of total protein)[c]			
		Glucose-6-phosphate dehydrogenase		Catalase	
		No treatment	Treatment with aspirin	No treatment	Treatment with aspirin
EG110	48	0.236 ± 0.009	0.261 ± 0.008	6.896 ± 0.157	$3.860 \pm 0.782^*$
EG110	72	0.186 ± 0.003	0.204 ± 0.033	8.570 ± 0.720	$3.710 \pm 1.022^*$

[a] EG110 is the MnSOD-deficient yeast strain.
[b] Cells were grown in rich medium containing the non-fermentable carbon source ethanol (YPE). Fifteen millimolar aspirin was added to medium and the pH adjusted to 5.5 with 1-M Trizma base. Protein extracts were prepared after 48 and 72 h of cultivation. Glucose-6-phosphate dehydrogenase and catalase activity were measured as described in Section 2.
[c] One unit of glucose-6-phosphate dehydrogenase activity corresponded to the amount of enzyme reducing 1 μmol of D-glucose 6-phosphate min^{-1} at 25 °C. One unit of catalase activity corresponded to decomposition of 1 μmol H_2O_2 min^{-1} at 20 °C. Values are the mean of at least three experiments ±SE.
* $P < 0.05$; treatment versus no treatment, two-tailed *t*-test.

Table 2
Levels of $NADP^+$ and NADPH in *S. cerevisiae* cells grown in the absence and presence of aspirin

Strains[a]	Media[b]	No treatment (nmol A_{600} $unit^{-1}$)			Treatment with aspirin[c] (nmol A_{600} $unit^{-1}$)[d]		
		NADPH	$NADP^+$	NADPH/$NADP^+$	NADPH	$NADP^+$	NADPH/$NADP^+$
EG103[§]	YPE	0.050 ± 0.002	0.007 ± 0.001	7.2 ± 1.2	0.049 ± 0.003	0.009 ± 0.002	6.2 ± 1.2
EG110[†]	YPE	0.055 ± 0.003	0.020 ± 0.002	2.8 ± 0.2	$0.039 \pm 0.001^{*}$	$0.014 \pm 0.001^{\#}$	2.8 ± 0.2
EG110[§]	YPE	0.050 ± 0.004	0.032 ± 0.001	1.6 ± 0.2	$0.017 \pm 0.001^{*}$	$0.012 \pm 0.002^{*}$	1.6 ± 0.3
EG110[§]	YEPG	0.077 ± 0.006	0.033 ± 0.003	2.4 ± 0.2	0.062 ± 0.001	0.035 ± 0.002	1.8 ± 0.1
EG110[§]	YPA	0.054 ± 0.001	0.018 ± 0.002	3.2 ± 0.5	0.056 ± 0.003	0.026 ± 0.003	2.5 ± 0.2

[a] EG110 is MnSOD-deficient whereas EG103 is the wild-type strain.
[b] Culture media containing enriched yeast extract, peptone-based medium with either 3% ethanol (YPE), 3% glycerol (YEPG), or 2% potassium acetate (YPA). Cells were harvested after 48 h ([†]) and 72 h ([§]) of cultivation and the levels of $NADP^+$ and NADPH were determined as described in Section 2.
[c] Fifteen millimolar aspirin was added to media and the pH adjusted to 5.5 with 1-M Trizma base, with the exception of YPA where the pH was already 5.8.
[d] The levels of $NADP^+$ and NADPH are expressed as nanomoles per A_{600} turbidimetric unit of culture, which is equivalent to 10^7 cells ml^{-1}. Values are the mean of at least three experiments ±SE.
[*] $P < 0.001$.
[#] $P < 0.05$; treatment versus no treatment, two-tailed *t*-test.

in the reducing power of these cells (Table 2), the levels of NADPH and $NADP^+$ remained much higher than when the cells were grown in ethanol medium. These much higher levels of NADPH and $NADP^+$ appear to protect the MnSOD-deficient cells against any adverse effect of aspirin on cell growth and survival.

3.4. Aspirin-treated MnSOD-deficient cells in YPE medium are characterized by a significant increase in GSSG level and a dramatic fall in GSH/GSSG ratio

To investigate further the redox status of the cells we measured GSH and GSSG levels. EG110 cells cultivated in YPE medium for 48 h showed a GSH level of 3.87 nmol A_{600} $unit^{-1}$ and a GSSG level of 0.032 nmol A_{600} $unit^{-1}$. After 72 h there was an increase of 23% in the GSH level, but no increase in the GSSG level, which led to a further increase in the GSH/GSSG ratio characteristic of proliferating cells (Table 3). GSH and GSSG levels in the wild-type EG103 cells were similar to those measured in EG110 cells, indicating a reducing intracellular environment.

Aspirin-treated EG110 cells cultivated for 48 h showed an increased GSSG level, although the GSH level was maintained. Thus, there was a fall of ~28% in the GSH/GSSG ratio on treatment with aspirin. After 72 h of cultivation, the GSH level remained the same as at 48 h, however, the GSSG level increased significantly, and this led to a threefold decrease in the GSH/GSSG ratio. In aspirin-treated EG103 cells, a near-doubling of the GSH level did not allow such a drastic fall in the GSH/GSSG ratio (Table 3).

At 72 h, the GSH and GSSG levels in EG110 cells were considerably higher when cultivated in glycerol

Table 3
Levels of GSH and GSSG in *S. cerevisiae* cells grown in the absence and presence of aspirin

Strains[a]	Media[b]	No treatment (nmol A_{600} $unit^{-1}$)			Treatment with aspirin[c] (nmol A_{600} $unit^{-1}$)[d]		
		GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
EG103[§]	YPE	3.63 ± 0.32	0.023 ± 0.00	159 ± 12	$7.24 \pm 1.27^{\#}$	$0.059 \pm 0.01^{\#}$	$123 \pm 4^{\#}$
EG110[†]	YPE	3.87 ± 0.22	0.032 ± 0.00	123 ± 11	3.89 ± 0.22	$0.045 \pm 0.01^{\#}$	$87 \pm 4^{\#}$
EG110[§]	YPE	4.77 ± 0.25	0.031 ± 0.00	156 ± 11	$3.80 \pm 0.22^{\#}$	$0.074 \pm 0.00^{*}$	$51 \pm 2^{*}$
EG110[§]	YEPG	10.03 ± 0.26	0.071 ± 0.01	143 ± 7	$16.16 \pm 0.65^{*}$	$0.177 \pm 0.01^{*}$	$91 \pm 3^{*}$
EG110[§]	YPA	11.83 ± 0.26	0.879 ± 0.03	13 ± 0.4	11.94 ± 0.53	$0.542 \pm 0.04^{**}$	$22 \pm 2^{\#}$

[a] EG110 is MnSOD-deficient whereas EG103 is the wild-type strain.
[b] Culture media containing enriched yeast extract, peptone-based medium with either 3% ethanol (YPE), 3% glycerol (YEPG), or 2% potassium acetate (YPA). Cells were harvested after 48 h ([†]) and 72 h ([§]) of cultivation and the levels of GSH and GSSG were determined as described in Section 2.
[c] Fifteen millimolar aspirin was added to media and the pH adjusted to 5.5 with 1-M Trizma base, with the exception of YPA where the pH was already 5.8.
[d] The levels of GSH and GSSG are expressed as nanomoles per A_{600} turbidimetric unit of culture, which is equivalent to10^7 cells ml^{-1}. Values are the mean of at least three experiments ±SE.
[*] $P < 0.001$.
[**] $P < 0.01$.
[#] $P < 0.05$; treatment versus no treatment, two-tailed *t*-test.

or acetate medium. On treatment with aspirin the GSH/GSSG ratio of cells grown in glycerol medium decreased significantly, by ~36% with respect to untreated cells because of greater increase in the GSSG relative to the GSH level. However, the GSH level increased by 61% and the cells remained fully viable. In acetate medium, the GSH/GSSG ratio increased significantly on treatment with aspirin because the GSSG level decreased considerably whilst the GSH level remained more or less the same (Table 3). The high GSH level in cells grown in these two media seems to be protective by maintaining a reducing intracellular environment as well as in ensuring proper functioning of essential catalytic and metabolic processes, rendering the cells viable.

4. Discussion

Our previous observations that aspirin, whilst exerting an antioxidant effect, induced apoptosis in MnSOD-deficient *S. cerevisiae* cells in YPE medium [4], urged us to investigate some metabolic processes and the intracellular redox state that may have led to cell death in the MnSOD-deficient cells.

Aspirin has been reported to acetylate several enzymes essential to major metabolic pathways [15]. Thus, we studied the effect of aspirin on glucose-6-phosphate dehydrogenase, the rate limiting enzyme of the pentose phosphate pathway reported to maintain constitutive levels of NADPH in yeast [16]. Treatment with aspirin did not inhibit the activity of glucose-6-phosphate dehydrogenase in MnSOD-deficient (Table 1) or wild-type strains (data not shown). This is in contrast to in vitro investigations, where it was demonstrated that aspirin acetylates yeast glucose-6-phosphate dehydrogenase, producing irreversible inactivation of the enzyme [17,18]. In our studies, treatment with salicylate, the major metabolite of aspirin, also inhibited cell growth (Fig. 1), indicating that loss in viability in the MnSOD-deficient cells is not a result of acetylation.

Pertinent for the stability and functioning of a large number of proteins is the maintenance of the cellular redox balance, in both the cytosol and subcellular compartments. This is ensured by the redox ratios $NADPH/NADP^+$ and GSH/GSSG, as well as the absolute GSH concentration [5,19]. Indeed mitochondria are dependent on cytosolic synthesis of GSH, which is transported to and recycled in the mitochondria [20,21]. Our results show that the reducing power, as measured by the $NADPH/NADP^+$ ratio, in MnSOD-deficient cells growing in ethanol medium was significantly lower than in wild-type cells having both Mn- and CuZnSOD (Table 2). This is quite conceivable since the mitochondrial respiratory chain generates ROS during growth on non-fermentable carbon sources, and the absence of MnSOD renders the cells more vulnerable to oxidative damage [22]. Although the $NADPH/NADP^+$ ratio in these cells was low, the NADPH pool was large enough to maintain a low GSSG level and high GSH/GSSG ratio, and thus sustain growth. The reducing power in MnSOD-deficient cells was higher when grown in glycerol or acetate medium, as reflected by the $NADPH/NADP^+$ ratio and high GSH levels (Tables 2 and 3), possibly due to the redirection of different metabolic pathways [23].

Cultivation of the yeast strains with aspirin caused a decrease in the $NADPH/NADP^+$ ratio, as seen with the MnSOD-deficient strain in glycerol or acetate medium, and the wild-type strain in ethanol medium (Table 2). Nevertheless, under these conditions the two strains were able to compensate for the altered redox state. The wild-type EG103 strain was able to compensate for this perturbation by increasing GSH production and thus maintaining the GSH/GSSG ratio at more than double the ratio of the MnSOD-deficient strain. Environmental stresses have previously been reported to increase the cellular GSH pool [24,25]. Additionally, the higher NADPH and GSH levels in the MnSOD-deficient cells when cultured in glycerol or acetate medium provide the cells with a greater reducing capacity. This seems to prevent loss in viability on treatment with aspirin, which is an antioxidant that scavenges ROS but does not serve as a reducing agent.

In ethanol medium, however, the $NADPH/NADP^+$ ratio, already at a critically low level in untreated MnSOD-deficient cells, remained the same upon treatment with aspirin, but viability was lost after 72 h of cultivation, since the cells were unable to maintain NADPH and $NADP^+$ levels. Similar findings are given by Petit et al. [26], who report depletion in NADPH in Jurkat T-cells before the onset of signs of apoptosis, after incubation with CD95-specific IgM antibody, αCD95, and propose NADPH depletion to be a major component of the apoptotic induction pathway. Additionally Pias and Aw [27] showed that diamide induced apoptosis in a mitotic competent, undifferentiated cell line, PC-12, due to a loss in redox balance, independently of ROS production, and this was exacerbated when levels of NADPH were reduced.

Loss in NADPH also led to an increase in the GSSG level, which resulted in a dramatic drop in the GSH/GSSG ratio in MnSOD-deficient cells. The GSH level after 72 h of cultivation remained the same as at 48 h. However, it was only half the GSH level in wild-type cells. Thus, although there was no significant shift in the $NADPH/NADP^+$ ratio after 72 h of cultivation with aspirin, the demand for NADPH superseded its production and affected negatively the GSH/GSSG ratio and cellular redox balance. Indeed, our results are in agreement with previous findings which report that compromised intracellular GSH concentrations render cells more vulnerable to an altered redox balance and lead

to growth arrest [28]. Whether the aspirin-treated cells proliferate or are committed to apoptosis depends on the severity of the redox shift, as seen with the MnSOD-deficient cells in ethanol medium.

Catalase activity in MnSOD-deficient cells in ethanol medium also decreased significantly with aspirin, possibly as a result of decreased protection of the enzyme by NADPH. Yeast catalases contain bound NADPH which serves to prevent catalase from oxidative damage and formation of an inactive state [29]. Depletion in NADPH has pleiotropic effects on the cell metabolism since NADPH is critical in regulating several metabolic pathways, maintaining reductive biosynthesis and the redox environment. It is the principal reductant of glutathione and thioredoxin which in turn maintain other sulphydryl groups in the reduced state, such as glutaredoxins. These antioxidants play an important role in maintaining the redox balance of the cell and allow the functioning of enzymes, transcription factors and membrane proteins [7,30].

It can thus be seen that cell viability is maintained by the complex interplay of metabolic processes with the redox environment. In the presence of aspirin, the cellular content of NADPH in MnSOD-deficient cells in ethanol medium decreased to a critically low level where the NADPH pool was probably too low to fulfill the biosynthetic requirements of the cell, and disruption of the redox balance committed the cells to apoptosis. The redox imbalance preceded apoptotic induction, possibly due to loss of mitochondrial reductants. Consequently, decreased reducing power may be linked to apoptotic induction by aspirin, independently of the level of ROS. It would seem that the protective effect of MnSOD, exerted in the isogenic wild-type cells, is related to the maintenance of the cellular reducing power which is essential for sustaining growth.

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