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ORIGINAL RESEARCH

Ascorbate and thiol antioxidants abolish sensitivity of yeast *Saccharomyces cerevisiae* to disulfiram

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Abstract Sensitivity of baker's yeast to disulfiram (DSF) and hypersensitivity of a mutant devoid of Cu, Zn-superoxide dismutase to this compound is reported, demonstrating that yeast may be a simple convenient eukaryotic model to study the mechanism of DSF toxicity. DSF was found to induce oxidative stress in yeast cells demonstrated by increased superoxide production and decrease of cellular glutathione content. Anoxic atmosphere and hydrophilic antioxidants (ascorbate, glutathione, dithiothreitol, cysteine, and *N*-acetylcysteine) ameliorated DSF toxicity to yeast indicating that oxidative stress plays a critical role in the cellular action of DSF.

Keywords Antioxidant · Disulfiram · Oxidative stress · Superoxide dismutase · *Saccharomyces cerevisiae* · Yeast

Abbreviations

ALDH	acetaldehyde dehydrogenase
ASC	ascorbate
CYS	cysteine
DDC	*N,N*-diethyldithiocarbamate
DHET	dihydroethidine
DSF	disulfiram
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GSH	reduced glutathione
GSSG	oxidized glutathione
MeDDC	*S*-methyl-*N,N*-diethyldithiocarbamate
MeDTC	*S*-methyl-*N,N*-diethylthiocarbamate
NAC	*N*-acetylcysteine

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Introduction

Disulfiram (DSF, Antabuse) is a drug used to support the treatment of chronic alcoholism by producing an extremely aversive reaction when taken in the presence of alcohol, and is thus considered a deterrent. DSF prevents conversion of acetaldehyde to the harmless acetic acid by blocking acetaldehyde dehydrogenase (ALDH, EC 1.2.1.10).

In the presence of reduced glutathione (GSH), disulfiram is rapidly reduced in vivo to *N,N*-diethyldithiocarbamate (DDC), which is methylated to form *S*-methyl-*N,N*-diethyldithiocarbamate (MeDDC). MeDDC is oxidized primarily to the intermediate metabolite MeDDC sulfine, which is ultimately converted to *S*-methyl-*N,N*-diethylthiocarbamate (MeDTC) sulfoxide, the proposed active metabolite of disulfiram in vivo,

and a small amount of MeDDC sulfoxide (Pike et al. 2001).

Mechanism of DSF action within a cell is complex and multi-level. DSF and its metabolite-DDC inhibit superoxide dismutase (SOD) in vitro and in vivo by copper chelation (Heikkila et al. 1976; Marikovsky et al. 2003; Kelner and Alexander 1986). Cells incubated with DSF show transient elevation of intracellular superoxide level, and progressive decrease of intracellular H_2O_2 level (Cen et al. 2002). Both DSF and DDC may also induce oxidative stress by shifting thiol redox balance. It was reported that DSF does not deplete total glutathione but significantly decreases the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio, by induction of glutathione oxidation (Cen et al. 2002; Burkitt et al. 1998; Namazi 2008). According to Grosicka-Maciag et al., DSF increases catalase and glutathione reductase activities, decreases non-Se-dependent glutathione peroxidase (GPx) activity, but does not change Se-dependent GPx in V79 fibroblasts. In contrast to other data, these authors did not reveal changes in SOD-1 and SOD-2 activities (Grosicka-Maciag et al. 2010). Several studies have suggested that DSF can induce apoptosis, DNA fragmentation, changes in mitochondrial membrane potential (Cen et al. 2002; Burkitt et al. 1998), and increase of protein carbonyl content (Grosicka-Maciag et al. 2010). Furthermore, DSF inhibits ATP hydrolysis and binds to substrate sites of several ABC transporters associated with multidrug resistance, blocking their activity (Sauna et al. 2004; Sauna et al. 2005). DSF metabolites like DDC can elevate copper level, leading to oxidative stress, protein damage, lipid peroxidation, and apoptosis in some cell types (Chen et al. 2001; Tonkin et al. 2004; Viquez et al. 2007), whereas MeDDC sulfoxide and MeDDC sulfine can inhibit the verapamil-stimulated ATPase activity of P-glycoprotein (Loo et al. 2004).

DSF, apart from treatment of alcoholism, has been examined as a potential drug effective in protection against *Giardia lamblia* (Nash & Rice 1998), *Trichomonas vaginalis* (Bouma et al. 1998), and leishmaniasis (Namazi 2008); in the therapy of candidiasis (Shukla et al. 2004); as a potential inhibitor of inflammation (Marikovsky et al. 2003); and in alternative therapy of some types of cancers (Cen et al. 2002; Sauna et al. 2005; Navrátilová et al. 2009; Wang et al. 2011). Khan et al. demonstrated antifungal activity of DSF against both yeast and filamentous fungi (Khan et al. 2007).

Previous studies have shown that antioxidants can reduce the cytotoxic effects of DSF treatment; for example, *N*-acetylcysteine (NAC) prevents DSF-induced apoptosis, augments cell viability and the GSH/GSSG ratio in human melanoma cells (Cen et al. 2002), and also prevents the increase in protein carbonyl content induced by disulfiram in V79 cells (Grosicka-Maciag et al. 2010). Dithiothreitol (DTT) completely prevents DSF-induced inhibition of ALDH at pH 7.5 in vitro; however, the inhibition induced by MeDTC is prevented by DTT only at pH 9.0 (Veverka et al. 1997). DTT protects also ATPase activity (Shukla et al. 2004).

In this paper, we show that mutant of yeast *Saccharomyces cerevisiae* deficient in Cu, Zn-superoxide dismutase (Sod1p), the enzyme decomposing superoxide anion, is hypersensitive to DSF. We demonstrate that several antioxidants such as ascorbate, cysteine, dithiothreitol, glutathione, and *N*-acetylcysteine abolish this sensitivity.

Material and methods

Chemicals

Disulfiram (tetraethylthiuram disulfide), CAS number 97-77-8, purum, ≥97%, was from Aldrich (Sigma-Aldrich, Poznan, Poland). A stock solution of DSF was freshly prepared in absolute ethanol (MERC, Germany) before each experiment. Dihydroethidine (DHET), FUN-1, and MitoTracker-Green stain were from Molecular Probes (Eugene, Oregon, USA). Monochlorobimane was from Fluka (Sigma-Aldrich, Poznan, Poland). Antioxidants and all other reagents were purchased from Sigma-Aldrich (Poznan, Poland). Components of culture media were from BD Difco (Becton Dickinson and Company, Spark, USA) except for glucose (POCh, Gliwice, Poland).

Yeast strains and growth conditions

Following yeast strains were used: wild-type SP4 MATα *leu1 arg4* (Bilinski et al. 1978), and Δ*sod1* mutant, isogenic to SP4, MATα *leu1 arg4 sod1::natMX* (Koziol et al. 2005). Yeast was grown in standard liquid YPD medium (1% yeast extract, 1% yeast bacto-peptone, and 2% glucose) on a rotary

shaker at 150 rpm or on solid YPD medium containing 2% agar, at a temperature of 30°C.

DSF toxicity assays

Liquid yeast cultures (200 μl of cell suspension containing 1×10^6 cells) with various concentrations of DSF (0–75 μM) were cultivated in a Bioscreen C (Oy Growth Curves Ab Ltd.) incubator with shaking at 30°C. Their growth was monitored turbidimetrically at 600 nm for 24 h (with measurements every 1 h).

For spotting tests, yeast exponential phase cultures were diluted to give suspensions of 10^7, 10^6, 10^5, and 10^4 cells/ml. Aliquots of 5 μl of each suspension were inoculated on solid YPD medium containing DSF (0–100 μM). Freshly prepared stock solution of DSF was added to sterile media, after cooling to approximately 50°C. In case of experiments under anaerobic conditions, cells were grown on YPD plates with or without 50 μM DSF in a desiccator under the atmosphere of 100% gaseous oxygen-free nitrogen (Linde Gaz, Cracow, Poland). Colony growth was inspected after 48 h.

In experiments involving antioxidants, cells were grown on liquid or solid YPD medium containing 50 μM DSF with various concentration of antioxidants, viz. ascorbate (ASC), cysteine (CYS), dithiothreitol, glutathione (GSH), *N*-acetylcysteine, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), or Trolox. In case of liquid medium, cell growth was inspected after 18 h, and on solid medium after 48 h.

Measurement of reactive oxygen species

Generation of superoxide was assessed with dihydroethidine at the final concentration of 18.9 μM (added from a stock solution was prepared in DMSO). Cells from exponential phase culture were centrifuged, washed twice, and suspended in 100 mM phosphate buffer, pH 7, containing 0.1% glucose and 1 mM ethylenediaminetetraacetic acid (EDTA) and DSF at concentrations of 0, 20, 50, and 100 μM at a density of 10^8 cells/ml. After 1, 2, and 3 h incubation cells were centrifuged, washed twice, and suspended in the same buffer. The kinetics of fluorescence increase, due to oxidation of fluorogenic probe, was measured using a Hitachi F2500 fluorescence spectrophotometer. Measurement conditions were: λ_{ex}= 518 nm and λ_{em}=605 nm; temperature of 30°C.

Fluorescence microscopy

Cells from exponential phase culture were centrifuged, washed twice, and suspended to a final density of 10^8 cells/ml in 100 mM phosphate buffer, pH 7, containing 0.1% glucose and 1 mM EDTA, and DSF at concentrations of 0, 20, 50, and 100 μM. After incubation for 3 h cells were pelleted by centrifugation and washed twice with PBS. The cellular content of glutathione was estimated with monochlorobimane according to a slightly modified method of Staleva et al. (Staleva et al. 2002). Briefly, cells were incubated with 30 μM monochlorobimane for 30 min and then fluorescence of the bimane-glutathione conjugates was observed using excitation wavelength of 480 nm. Mitochondria were stained with MitoTrackerGreen and metabolic activity of yeast cells was assessed with FUN-1 according to the manufacturer's protocols (Molecular Probes). The fluorescence pictures were taken with an OLYMPUS BX-51 microscope equipped with a DP-72 digital camera.

Statistical analysis

The results represent mean ± SD from at least three independent experiments. Statistical analysis was performed using the SPSS 17.0 software. The statistical significance of differences between means of treated samples compared to untreated control was estimated using one-way ANOVA and the Dunnet post hoc test. The differences between means of two yeast strains compared were evaluated using the *t* test for independent samples. Values were considered significant if $P<0.05$.

Results

A mutant defective in Sod1p is hypersensitive to DSF

Exposure of yeast in both liquid and solid medium to DSF resulted in inhibition of growth in a concentration-dependent manner, Δ*sod1* mutant showing much higher sensitivity (Fig. 1a–c). Complete inhibition of growth under the influence of DSF in liquid medium could be observed at a concentration of 75 μM for wild

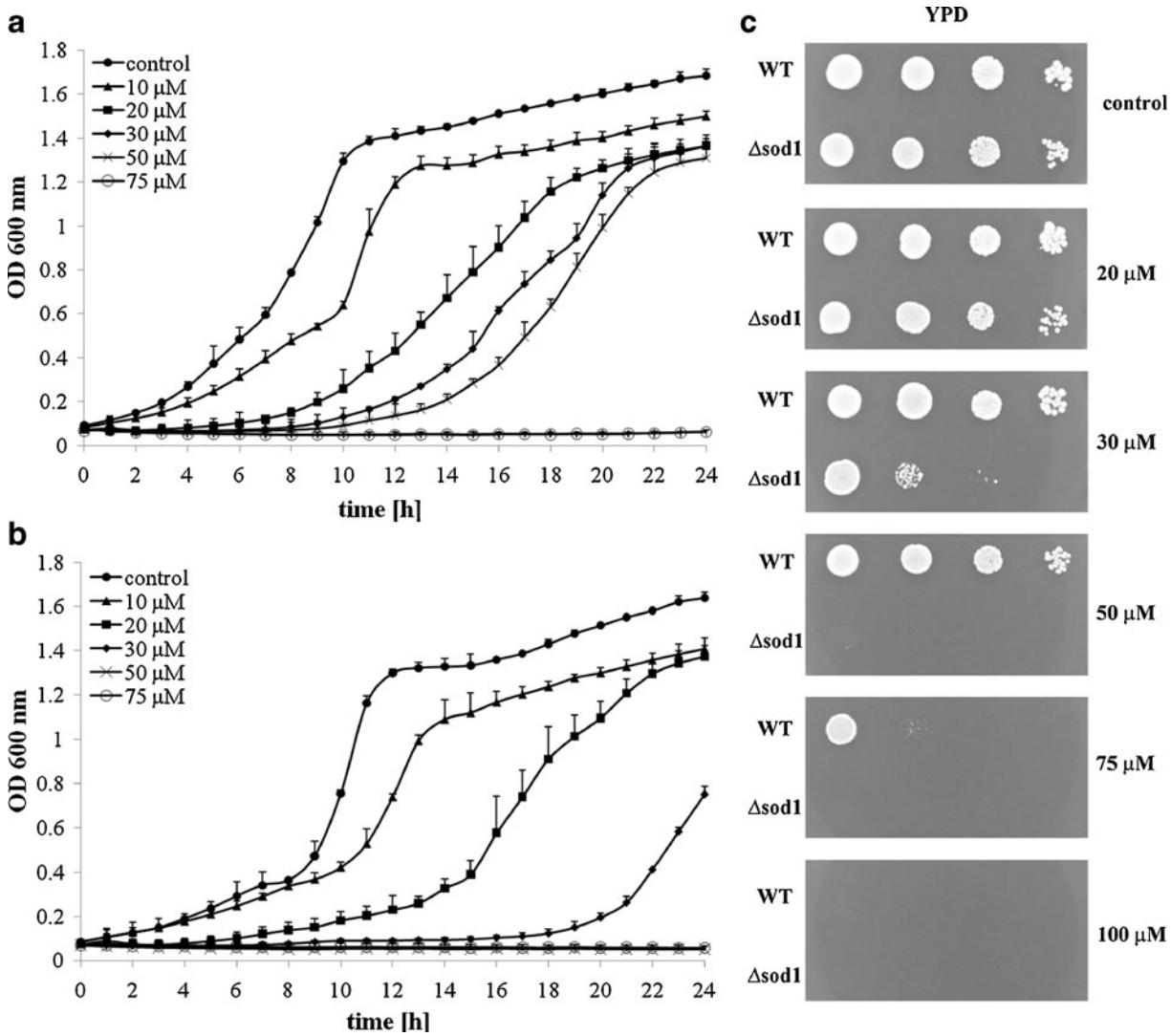

Fig. 1 Effect of DSF (0–100 μM) on yeast cell growth on liquid and solid media. Kinetics of growth of WT strain SP4 (**a**) and Δ*sod1* mutant (**b**) was monitored turbidimetrically at 600 nm every 1 h for 24 h. Data are presented as mean±SD from three independent experiments; **c** in spotting tests colony growth was recorded after 48 h. Successive spots contained initially 50,000, 5,000, 500, and 50 cells

type (WT) and of 50 μM for Δ*sod1* mutant. However, a 50% decrease in survival of Δ*sod1* mutant was noticed already at 30 μM concentration of DSF. Lower concentrations of DSF tested (10–50 μM in the case of WT, and 10–20 μM in the case of Δ*sod1*) did not cause significant inhibition of growth after 24 h (mortality less than 25% compared with control), but clearly changed the course of growth curves (Fig. 1a, b). On solid medium, complete inhibition of growth was apparent at the concentration of 100 μM DSF in the case of WT and of 50 μM DSF in the case of Δ*sod1* mutant. Partial inhibition of growth of WT could be observed from the concentration of 75 μM, and in the case of Δ*sod1* mutant of 30 μM of DSF (Fig. 1c). Both in liquid and on solid medium, there was no effect of introduced amounts of ethanol (DSF solvent) on the growth of yeast cells (data not shown).

DSF induces oxidative stress in yeast cells

Treatment of Δ*sod1* mutant cells with 20, 50, and 100 μM DSF for 1, 2, and 3 h induced a significant increase in the rate of ROS generation estimated with

DHET (Fig. 2). In the case of WT and Δ*sod1* cells we observed an increased oxidation of DHET after 2 and 3 h incubation in the buffer. This effect was especially significant in the case of Δ*sod1* mutant cells. Ethanol (disulfiram solvent) did not cause oxidation of the probe, in the amounts introduced with DSF (data not shown). Yeast cells showed a concentration-dependent decrease in the GSH content after exposure to different concentrations of disulfiram. Changes of intracellular GSH content were greater for Δ*sod1* than for WT cells (Fig. 3a). Microscopic visualization showed that exposure of yeast cells to DSF in the concentration range of 20–100 μM caused a significant decrease of metabolic activity, increase in cell death frequency, changes of morphology, and mitochondria disintegration, observed especially for Δ*sod1* mutant cells (Fig. 3b, c).

Antioxidants and anoxia abolish sensitivity of yeast to DSF

The inhibition of growth of Δ*sod1* mutant cells in the presence of DSF may be overcome by addition of low molecular, hydrophilic antioxidants to growth medium (Fig. 4a, b). In the case of liquid medium, complete abolition of sensitivity of both yeast strains to 50 μM DSF was reached at 5 mM ASC, CYS, GSH, NAC, and at 2.5 mM DTT. No protective effect was observed for 0.5 mM Tempol and 1 mM Trolox, and a slight potentiation of the effect of DSF on WT cells was even noted for 0.5 mM Tempo (Fig. 4a). A similar effect was observed on solid medium: complete abolition of the effect of DSF was achieved for ASC, CYS, GSH, and NAC (1 and 5 mM), and partial amelioration for DTT (0.1, 1, and 5 mM) while Tempo, Tempol, and Trolox were ineffective (Fig. 4b). Anoxic atmosphere, similarly to antioxidants, led to complete abolishment of growth inhibition of Δ*sod1* mutant cells in the presence of DSF (Fig. 4c).

Discussion

Despite of the fact that DSF is mainly known as an inhibitor of aldehyde dehydrogenase, it has also other effects on cells. It has been reported that DSF may induce oxidative stress. This mechanism of DSF action was examined in several mammalian cell lines

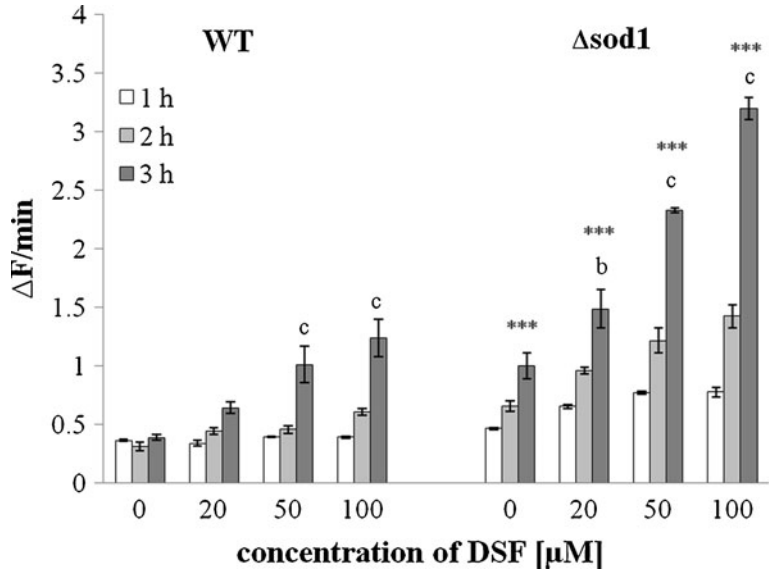

Fig. 2 Superoxide formation in yeast cells after treatment with DSF. Cells were suspended in 0.1 M phosphate buffer, pH 7.0, containing 0.1% glucose and 1 mM EDTA and treated with various concentrations of DSF for 1, 2, and 3 h. Superoxide formation was estimated by the rate of fluorescence increase due to DHET oxidation within cells. Data represent mean ± SD from three independent experiments, *asterisks* denote statistical significance with respect to wild-type yeast ***$P<0.001$, estimated by the *t* test for independent samples. The letters *b* and *c* indicate values significantly different from untreated control for the same yeast strain using ANOVA and Dunnet post hoc test at *b* $P<0.01$ and *c* $P<0.001$, respectively. Statistical analysis was performed only for samples incubated for 3 h with or without DSF

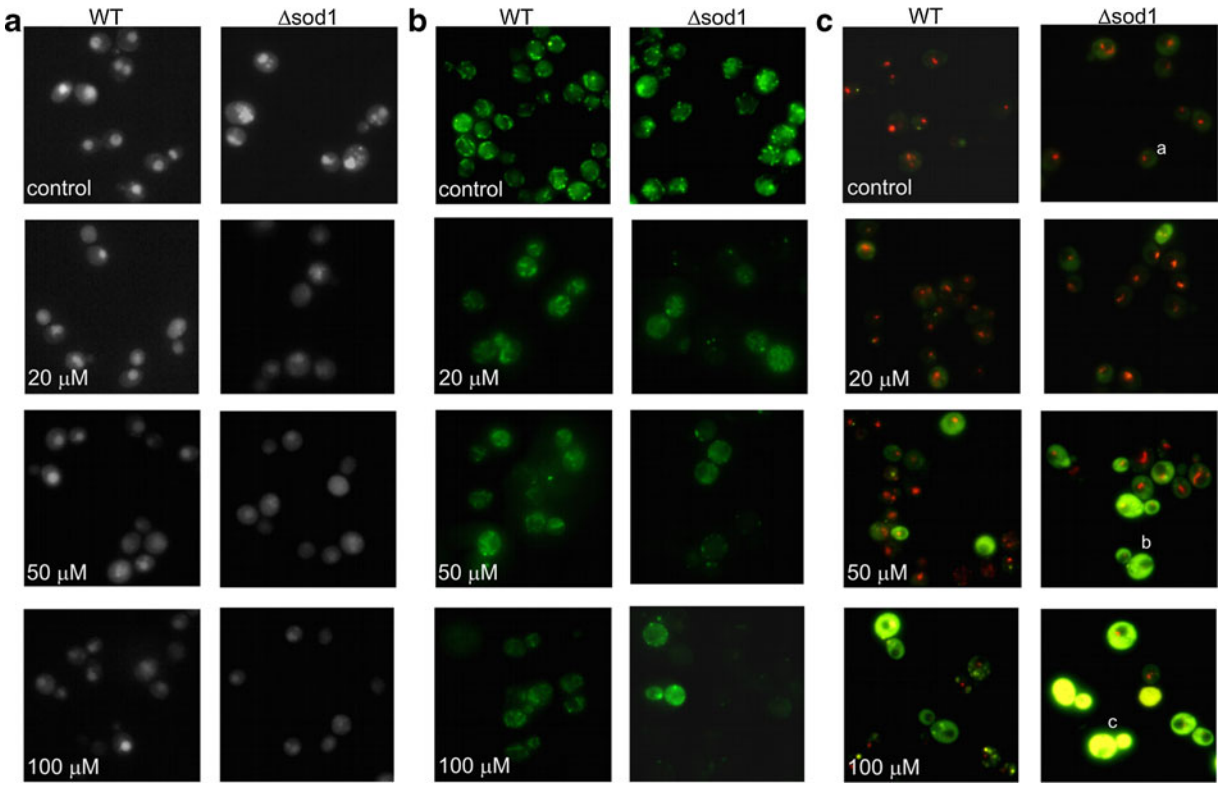

Fig. 3 Effect of DSF (0–100 μM) on yeast cells. **a** Changes in GSH content estimated with monochlorobimane. **b** Changes of mitochondria morphology, cell staining with MitoTrackerGreen. **c** Changes of metabolic activity, cell staining with FUN-1 (*a*—metabolically active cell; *b*—metabolically inactive cell; *c*—dead cell)

(Cen et al. 2002; Grosicka-Maciag et al. 2010; Chen et al. 2001). In this paper, we show that also yeast *S. cerevisiae* may provide new information on the mechanisms of cellular effects of DSF.

Yeast *S. cerevisiae* is widely accepted as a model eukaryotic organism. It has a number of attributes that make it attractive as an experimental system for studies of cell physiology, genetics, and cellular response to various environmental stresses. Despite its simplicity yeast cells are similar to higher eukaryotes in their biochemistry and physiology. Many studies confirm the usefulness of yeast for testing toxicity of xenobiotics including drugs (Yasokawa et al. 2008; Letavayová et al. 2008; Van der Heggen et al. 2010). Yeast can be grown under hypoxia or even complete anoxia, which makes yeast cells especially useful for studies of oxidative stress-related mechanisms. Their culture is much cheaper in comparison with mammalian cells. Furthermore, viable disruptants of all genes of *S. cerevisiae* are easily available which can allow for screening of genes whose products are important for cellular effects of xenobiotics.

Our results demonstrate that yeast cells lacking Cu, Zn-superoxide dismutase (Sod1p), the enzyme removing superoxide anion, are hypersensitive to DSF. DSF at a concentration of 30 μM caused an approximate 50% growth inhibition both in liquid and on solid medium, and completely inhibited growth of Δ*sod1* mutant cells at a concentration of 50 μM (Fig. 1b, c). We demonstrated that WT strain is also sensitive to DSF but growth inhibition is observed at higher concentrations (e.g., 75 μM of DSF; Fig. 1a, c). It is worth noting that almost the same concentrations were used in experiments with *S. cerevisiae* Δ*pso2* mutant (0–30 μM DSF) (Brendel et al. 2010), *C. albicans* (0–100 μM DSF) (Shukla et al. 2004), and astrocytes (0–100 μM DSF) (Chen et al. 2001). Thus, the sensitivity of very different cells to DSF is similar which may well justify the use of yeast cells in studies of the mechanism of action of this compound.

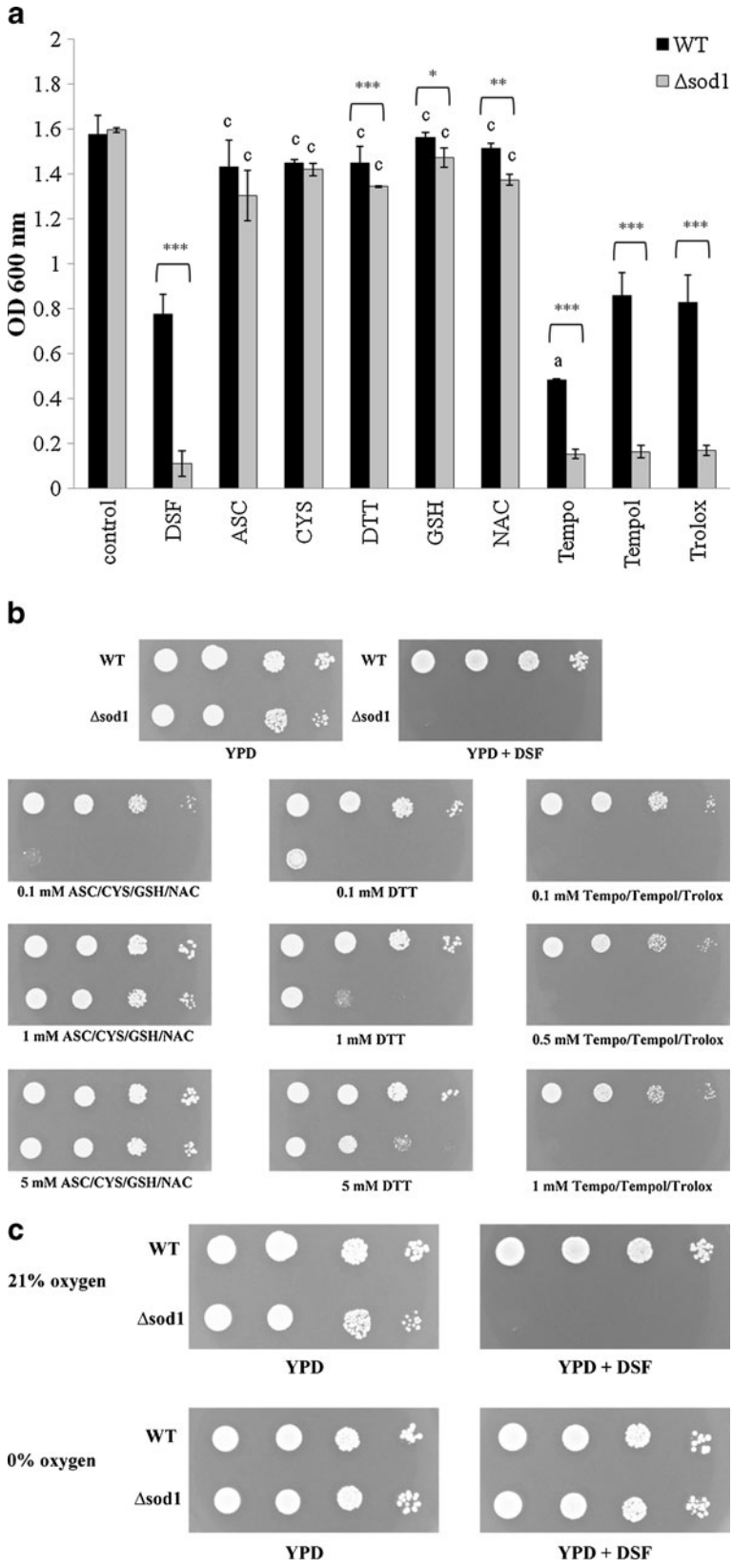

Fig. 4 Effect of antioxidants and anoxic atmosphere on yeast cell growth inhibition induced by 50 μM DSF. **a** Yeast cells were cultured in liquid medium with antioxidants (5 mM ASC, CYS, GSH, NAC; 2.5 mM DTT; 0.5 mM Tempo, Tempol and 1 mM Trolox) and growth rate was monitored turbidimetrically after 18 h. Data represent mean ± SD from three independent experiments, *asterisks* denote statistical significance with respect to wild-type yeast *$P<0.05$, **$P<0.01$, ***$P<0.001$, estimated by the *t* test for independent samples. The letters *a* and *c* indicate significantly different values from DSF treated sample with the same yeast strain using ANOVA and Dunnet post hoc test at *a* $P<0.05$, *c* $P<0.001$, respectively. **b** Yeast cells were grown on solid medium with the same antioxidants. **c** Yeast cells were grown on solid medium under anoxic atmosphere. For spotting tests colony growth was recorded after 48 h. Successive spots contained 50,000, 5,000, 500, and 50 cells

Our results show that DSF causes oxidative stress in yeast cells by significantly increasing superoxide generation (Fig. 2), and depletion of glutathione (Fig. 3a). Longer incubation (3-h) with 50 and 100 μM DSF causes also morphological changes, disintegration of mitochondria (Fig. 3b), and decrease of metabolic activity of cells (Fig. 3c). Mitochondria are known to be an important target for many oxidants and dynamic changes in their morphology may be accompanied by loss of mitochondrial membrane potential. Furthermore, these alterations as well as disintegration of mitochondria may be a trigger for apoptosis (Eisenberg et al. 2007).

The potential mechanism of DSF toxicity may be linked to the copper chelating activity of this compound. Though DSF does not inhibit superoxide dismutase in vitro, it decreases the activity of this enzyme in vivo, apparently due to reduction to diethyldithiocarbamate (DDC), a strong SOD inhibitor (Forman et al. 1980). In erythrocytes, DSF was found to oxidize GSH to GSSG and inhibit SOD, and these effects were ascribed to reduction of this compound to DDC as well (Kelner & Alexander 1986). Reduction of DSF to DDC and inhibition of Cu, Zn-superoxide dismutase leading to increase in superoxide steady state level and oxidation of glutathione seems also to be a plausible mechanism contributing to the effects of this compound in yeast.

The role of oxidative stress in the toxicity of DSF is confirmed by elimination of hypersensitivity of Δ*sod1* mutant cells to this compound under anoxia (Fig. 4c), and by the protection offered by some hydrophilic antioxidants: ascorbate, reduced glutathione, dithiothreitol, cysteine, and *N*-acetylcysteine (Fig. 4a, b). Amelioration of the effects of DSF by DTT and NAC has been reported previously for *C. albicans* (Shukla et al. 2004) and Chinese hamster fibroblast cells (Grosicka-Maciag et al. 2010). Protective effects of ascorbate and other hydrophilic antioxidants on DSF-induced oxidative stress have not been well studied. Yeast seems to be a useful eukaryotic model for studies of these effects at the cellular level.

Interestingly, some of antioxidants used like Tempo, Tempol, and Trolox had no protective effect (Fig. 4a, b). The same pattern of antioxidant action was found when studying rescue of Δ*sod1* mutant cells from other oxidants including *tert*-butyl hydroperoxide, cumene hydroperoxide, menadione, juglone, hypochlorite, chlorite, oxytetracycline, acrolein, acrylamide, and hypertonic stress (Koziol et al. 2005; Lewinska et al. 2004; Kwolek-Mirek et al. 2009; Kwolek-Mirek et al. 2011), which suggests that DSF induces oxidative stress by a mechanism similar to that of other oxidants.

In summary, our data show that yeast *S. cerevisiae* is sensitive to DSF. We demonstrate the important role of Cu, Zn-superoxide dismutase (Sod1p) in protection against DSF-induced oxidative stress. Hydrophilic antioxidants: ascorbate, cysteine, reduced glutathione, ditiothreitol, and *N*-acetylcysteine can abolish DSF-induced toxicity in yeast cells. The observation that not only thiol antioxidants but also ascorbate can protects from DSF toxicity may be useful in further studies and be of importance for the anti-alcoholic therapy in humans.

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