NO-mediated apoptosis in yeast

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Summary

Nitric oxide (NO) is a small molecule with distinct roles in diverse physiological functions in biological systems, among them the control of the apoptotic signalling cascade. By combining proteomic, genetic and biochemical approaches we demonstrate that NO and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are crucial mediators of yeast apoptosis. Using indirect methodologies and a NO-selective electrode, we present results showing that H_2O_2-induced apoptotic cells synthesize NO that is associated to a nitric oxide synthase (NOS)-like activity as demonstrated by the use of a classical NOS kit assay. Additionally, our results show that yeast GAPDH is a target of extensive proteolysis upon H_2O_2-induced apoptosis and undergoes S-nitrosation. Blockage of NO synthesis with N_ω-nitro-L-arginine methyl ester leads to a decrease of GAPDH S-nitrosation and of intracellular reactive oxygen species (ROS) accumulation, increasing survival. These results indicate that NO signalling and GAPDH S-nitrosation are linked with H_2O_2-induced apoptotic cell death. Evidence is presented showing that NO and GAPDH S-nitrosation also mediate cell death during chronological life span pointing to a physiological role of NO in yeast apoptosis.

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Key words: Yeast apoptosis, Nitric oxide, S-nitrosation, Glyceraldehyde-3-phosphate dehydrogenase, L-arginine, Reactive oxygen species

Introduction

Nitric oxide (NO) is a highly diffusible free radical with dichotomous regulatory roles in numerous physiological and pathological events (Ignarro et al., 1987; Nathan, 1992) being recognized as an intra- and inter-cellular signalling molecule in both animals and plants (Delledonne, 2005). Diverse cellular functions can be directly or indirectly affected by NO through posttranslational modification of proteins, of which the most widespread and functionally relevant one is S-nitrosation, defined as the covalent attachment of NO to the thiol side chain of a cysteine (Cys) (Hess et al., 2005). NO also controls the apoptotic signalling cascade by regulating the expression of several genes, mitochondrial dysfunction, and caspase activity/activation (Brune et al., 1995; Kroncke et al., 1995). Nonetheless, the mechanisms underlying the NO-mediated inhibition of apoptosis are not clearly understood, although it is well known that S-nitrosation is a crucial event to permanently maintain human caspases in an inactive form (Choi et al., 2002). Recent work demonstrated that like mammalian caspases, metacaspases, which are apoptosis-executing caspase-like proteases in yeast (Madeo et al., 2002) and plants (Suarez et al., 2004), can be kept inactive through S-nitrosation of one Cys residue (Belenghi et al., 2007). Nevertheless, a second catalytic Cys residue, highly conserved in all known metacaspases but absent in all members of caspases, can rescue the first S-nitrosated catalytic site even in the presence of high NO levels (Belenghi et al., 2007). In addition, S-nitrosation has been shown to regulate the function of an increasing number of intracellular proteins (Stamler et al., 2001), among them glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme that when S-nitrosated translocates to the nucleus, triggering apoptosis in mammalian cells (Hara et al., 2005).

The origin of NO in yeast cells is still a matter of debate essentially because of the lack of mammalian nitric oxide synthase (NOS) orthologues in the yeast genome, as previously observed in plants. Recently, Castello and coworkers (Castello et al., 2006) showed that yeast cells are capable of producing NO in mitochondria under hypoxic conditions. This production is nitrite dependent through cytochrome c oxidase and is influenced by YHb, a flavohaemoglobin NO oxidoreductase (Castello et al., 2006). Furthermore, results from our group showed that treatment of yeast cells with menadione leads to NO production dependent on intracellular L-arginine levels, suggesting the existence of an enzyme with NOS-like activity (Osorio et al., 2007). Supporting the relevance of NO in yeast physiology and pathophysiology is the presence of various cellular defences against nitrosative stress. Several molecules, such as peroxiredoxins, thioredoxins and the flavohaemoglobin, prompt yeast cells to face nitrosative stress (Liu et al., 2000; Wong et al., 2002). Moreover, when nitrosative stress is exogenously imposed it is sufficient to inactivate GAPDH

(Sahoo et al., 2003). However, the role of NO and its relevance in yeast apoptosis has never been explored.

Using proteomic, genetic and biochemical approaches we found evidence suggesting the intervention of NO and GAPDH in yeast H_2O_2-activated apoptotic pathway. NO production upon H_2O_2 treatment is dependent on intracellular L-arginine content and contributes to the generation of intracellular reactive oxygen species (ROS). GAPDH, the levels of which increase in total cellular extracts of H_2O_2-induced apoptotic cells, becomes fragmented and S-nitrosated, possibly acting as an apoptotic trigger. Chronologically aged cells also display increased NO production and GAPDH S-nitrosation, as well as a correlation between intracellular levels of superoxide anion and NO production, thereby suggesting a physiological role of NO in the signalling of yeast apoptosis.

Results

H_2O_2, as a ROS, triggers a stress response in *Saccharomyces cerevisiae* by activating a number of stress-induced pathways that might lead to alterations of gene expression, protein modification and translocation, growth arrest or apoptosis. Using 2-D gel electrophoresis coupled to mass spectrometry we analyzed both the mitochondrial and total cellular proteome of H_2O_2-induced apoptotic cells, identifying increased levels of several stress-related proteins (Fig. 1, Table 1). An acidic form of the thiol-specific peroxiredoxin, Ahp1p (Lee et al., 1999), previously described as the active form (Prouzet-Mauleon et al., 2002), and two spots of the thioredoxin peroxidase, Tsa1p (Garrido and Grant, 2002), were induced upon H_2O_2 treatment (Fig. 1, Table 1). Under the same conditions, increased levels of two mitochondrial stress-related proteins, Prx1p, a thioredoxin peroxidase (Pedrajas et al., 2000), and Ilv5p, required for the maintenance of mitochondrial DNA (Zelenaya-Troitskaya et al., 1995) were also detected (Fig. 1, Table 1).

NO is synthesized by an L-arginine-dependent process during H_2O_2-induced apoptosis

Taking into account that molecules such as peroxiredoxins and thioredoxins are known to play a role against both oxidative and nitrosative stress in several organisms, including yeast (Barr and Gedamu, 2003; Missall and Lodge, 2005; Wong et al., 2002; Wong et al., 2004), we questioned whether H_2O_2 might be inducing nitrosative stress due to an endogenous production of NO. Therefore, we performed several experiments in order to measure NO production in yeast cells dying by an apoptotic process triggered by H_2O_2. Given that NO is a diffusible free radical rapidly oxidized to nitrate and nitrite (Palmer et al., 1987), an indirect measurement of NO production by monitoring nitrate and nitrite formation was performed. The obtained results supported the hypothesis of NO production since nitrate concentrations increased upon exposure to H_2O_2 (Fig. 2A). Additionally, flow cytometric quantification of cells stained with the NO indicator 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM) diacetate revealed that a high percentage of H_2O_2-treated cells contain high NO and/or reactive nitrogen species (RNS) levels, which decreased when a non-metabolized L-arginine analogue, N_ω-nitro-L-arginine methyl ester (L-NAME), was added (Fig. 2B). Nonetheless, Balcerczyk and coworkers (Balcerczyk et al., 2005) have shown that NO quantitative determination by DAF-FM in the presence of ROS is overestimated, indicating that DAF-FM is a fairly specific NO probe. Thus, to support the hypothesis of NO production in H_2O_2-apoptosing cells we investigated NO synthesis in vivo using a NO-selective electrode (AmiNO-700). After H_2O_2 stimulus, yeast cells produced NO (Fig. 2C), the concentration of which increased in the medium following sigmoid-type kinetics. In fact, NO production was shown to be dependent of H_2O_2 concentration as indicated by the rate of NO production inferred from the curves during the initial linear periods (Fig. 2D). These results were further confirmed using a different NO-selective electrode (ISO-NO; World Precision Instruments; data not shown). Moreover, the results obtained with the NO-selective electrode supported that NO synthesis is L-arginine dependent

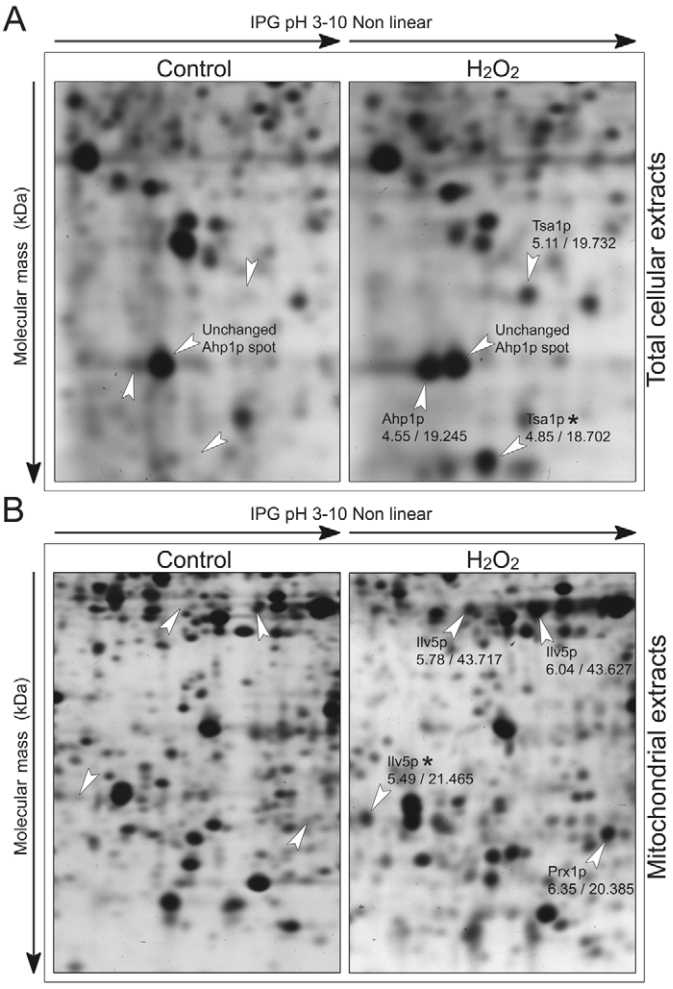

Fig. 1. The levels of stress response proteins are increased in both total and purified mitochondrial extracts from H_2O_2-induced apoptotic cells. Comparison of protein expression levels in total cellular (A) and purified mitochondrial extracts (B) of untreated (control) and H_2O_2-treated wild-type *S. cerevisiae* cells. Selected regions of the 2-D gel (isoelectric point/molecular mass) are shown enlarged and the position of altered protein spots are marked with an arrowhead. Putative protein fragments are marked with an asterisk. The apparent isoelectric points and molecular masses of the proteins were calculated with Melanie 3.0 (GeneBio) using identified proteins with known parameters as references.

Table 1. Proteins of mitochondrial and total cellular extracts with detected expression changes upon H_2O_2 treatment

Proteins identified	Spots	Function	Expression level*	Theoretical pI/mol. mass (kDa)	Experimental pI/mol. mass (kDa)
Total cellular proteome					
Stress response					
Ahp1	1	Thiol-specific peroxiredoxin	Up	5.01/19.115	4.55/19.245
Tsa1	2	Thioredoxin peroxidase	Up	5.03/21.458	4.85/18.702
			Up		5.11/19.732
Carbohydrate metabolism					
Adh1	1	Alcohol dehydrogenase	Up	6.26/36.692	5.68/42.191
Eno2	2	Enolase II	Up	5.67/46.783	5.45/45.268
			Up		5.97/43.214
Tdh2	1	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 2	Up	6.49/35.716	5.89/18.980
Tdh3	5	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	Up	6.46/35.747 (5.83/29.513)	5.57/31.420
			Up		5.54/20.168
			Up		5.78/20.188
			Up		5.72/19.118
			Up		5.60/37.475
Amino acid biosynthesis					
His4	1	Involved in histidine biosynthesis	Down	5.18/87.721	5.15/97.957
Unknown function					
Ycl026c-b	1	Hypothetical protein	Up	6.43/20.994	6.05/19.989
Mitochondrial proteome					
Ilv5	3	Acetohydroxyacid reductoisomerase	Up	9.10/44.368 (6.31/39.177)	5.49/21.465
			Up		6.04/43.627
			Up		5.78/43.717
Prx1	1	Mitochondrial peroxiredoxin	Up	8.97/29.496	6.35/20.385
Tdh3	1	Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	Up	6.46/35.747 (5.83/29.513)	6.44/37.008
Ycl026c-b	1	Hypothetical protein	Up	6.43/20.994	6.14/19.749

*An expression change was considered significant if the intensity of the corresponding spot differed reproducibly more than threefold.
Protein function was obtained from SGD (http://www.yeastgenome.org/). Theoretical pI (isoelectric point) and molecular mass (kDa) were calculated with the Compute pI/kDa tool (http://ca.expasy.org/tools/pi_tool.html).

since pre-incubation with L-NAME or D-arginine partially inhibited its synthesis (Fig. 2C). T80, time at which NO concentration is 80% of the maximal concentration, was 368.81 seconds for 4 mM of H_2O_2 and 384.52 and 403.38 seconds when cells were pre-incubated with D-arginine or L-NAME, respectively. Given the evidence supporting the synthesis of NO being dependent on L-arginine, we decided to use a classical method to detect a putative nitric oxide synthase (NOS)-like activity. Using a NOS assay kit that measures the formation of [^{3}H]citrulline from L-[^{3}H]arginine, we observed that H_2O_2-induced apoptotic yeast cells increased NOS-like activity in a H_2O_2 dose-dependent manner, concurrent with the previously observed NO production (Fig. 3A). Additionally, analysis of intracellular amino acid concentrations in yeast cells revealed that upon treatment there was a twofold increase in L-arginine concentration, which might be crucial for NO production upon H_2O_2-induced apoptosis (Fig. 3B). In fact, pre-incubation of cells grown in SC medium with L-arginine is sufficient to increase their susceptibility to H_2O_2 (data not shown). However, pre-incubation of cells with tyrosine, methionine, or glutamine, whose intracellular concentrations were also found to increase after H_2O_2 treatment (Fig. 3B), did not increase cellular susceptibility to this oxidative agent (data not shown). Moreover, results showing that pre-incubation with L-NAME rendered yeast cells resistant to H_2O_2 (Fig. 4A), further supported the hypothesis that NO synthesis occurs during, and accounts for, H_2O_2-induced apoptosis. In fact, cellular protection conferred by L-NAME was shown to be specific since it did not result in a cell-death-resistant phenotype when challenged with acetic acid (Fig. 4B). Also, rather than having a protective effect on cells dying by Bax heterologous expression, L-NAME actually increases Bax toxicity (Fig. 4C). In accordance with the specificity of NO production during H_2O_2-induced apoptosis, pre-incubation with L-NAME dramatically decreased the intracellular levels of ROS (Fig. 4D). Supporting the suggested correlation between NO production and the increase of ROS, cell treatment with the NO donor DETA/NO, previously used to induce nitrosative stress in yeast cells (Horan et al., 2006), resulted in intracellular ROS accumulation (data not shown).

Altogether our results demonstrated that upon H_2O_2-induced apoptosis, NO is produced in yeast cells by an L-arginine-dependent mechanism pointing to the requirement of a yet unknown protein with a NOS-like activity. Moreover, NO is presented herein as an important apoptotic regulator that correlates with the intracellular ROS levels generated during H_2O_2-induced apoptosis.

NO is produced during chronological life span leading to an increase of superoxide anion levels

In order to unravel the possible role of NO in a physiological scenario of yeast apoptosis, we evaluated its production during chronological life span. Our results demonstrated that chronologically aged cells (10 days) display more than a

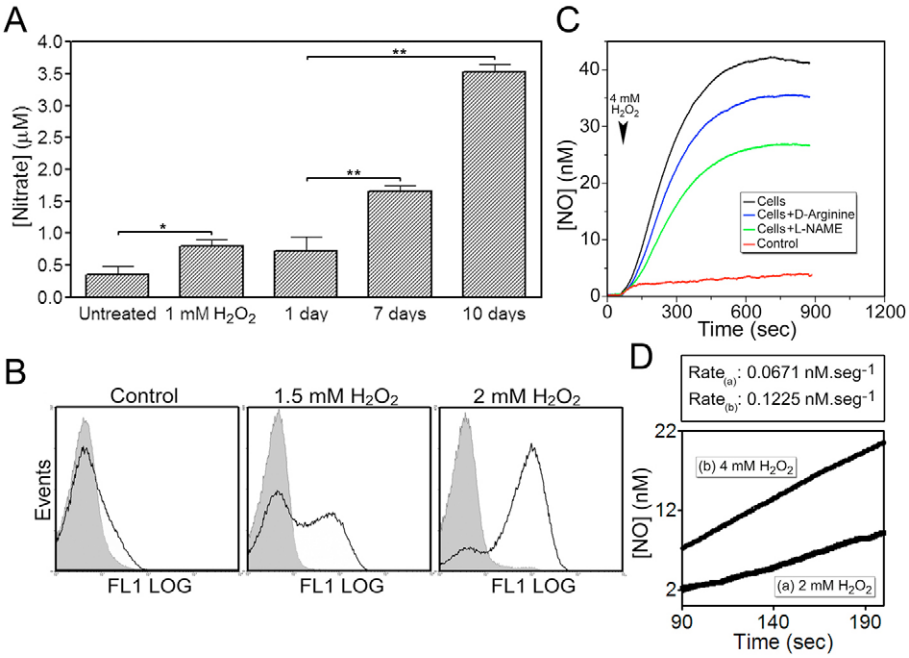

Fig. 2. Yeast cells synthesize NO upon apoptosis induction, which is dependent on L-arginine. (A) NO production in untreated, H_2O_2-treated and chronologically aged cells was indirectly assessed through measurement of nitrite and nitrate concentrations as described in Materials and Methods. *P≤0.05 versus untreated cells, **P≤0.03 versus 1-day-old cells; t-test, n=3. (B) NO production in untreated and H_2O_2-treated (1.5 and 2.0 mM) cells assessed by flow cytometric quantification of cells stained with the NO indicator DAF-FM diacetate, in the absence (white area under the peak) or presence (shaded area) of the non-metabolized L-arginine analogue L-NAME. The data are presented in the form of frequency histograms displaying relative fluorescence (x axis) against the number of events analyzed (y axis). (C) Direct measurement of L-arginine-dependent NO production upon H_2O_2-induced apoptosis. NO production was recorded with a NO-selective electrode (AmiNO-700) upon addition of 4 mM H_2O_2 to 5×10^8 wild-type cells (black line) or to wild-type cells pre-incubated with the non-metabolized D-arginine (blue line) or L-NAME (green line). A control experiment without cells was also recorded and is represented as a red line. (D) Rate of NO production is H_2O_2 dependent. 2 mM or 4 mM of H_2O_2 was added to 5×10^8 wild-type cells and NO production assessed using the NO-selective electrode (AmiNO-700). Data presented correspond to the linear part of the NO production curve. Rate of NO production was calculated from the slope.

fivefold increase in NO generation, as indirectly determined by an increase in nitrate concentrations, which directly correlated with aging time (Fig. 2A). Given the limitations of assessing NO production by a NO-selective electrode with a chronic stimulus such as aging, and aiming to address the consequences of the indirect observation of NO production, we also evaluated distinct cellular parameters in the presence of oxyhaemoglobin (OxyHb), a compound that scavenges NO and is considered a major route of its catabolism (Kelm et al., 1996; Pietraforte et al., 1995; Wennmalm et al., 1992), representing a gold standard test for the involvement of NO in a biological process (Ignarro et al., 1987; Joshi et al., 2002). Cells in the presence of OxyHb revealed a faster growth (Fig. 5A) and a delay in cell death induced during chronological life span (Fig. 5B), both of which are OxyHb dose dependent.

Superoxide anion, which is generated during chronological life span, is known to play a major role in the age-associated death of yeast and other eukaryotic cells (Fabrizio et al., 2004). In mammalian cells, superoxide anion interacts with NO leading to the formation of peroxynitrite (Packer et al., 1996), a RNS that enhances mitochondrial dysfunction, triggering an increased production of intracellular ROS levels (Zamzami et al., 1995). Following this line of thought, we evaluated the intracellular levels of superoxide anion during chronological life span in the presence of OxyHb. Our results demonstrated a decrease in superoxide anion production during chronological life span, which was inversely correlated to OxyHb concentrations (Fig. 5C). Overall, these results point to the occurrence of NO synthesis during chronologic life span and a role for NO during physiological apoptotic cell death. Moreover, NO is suggested to mediate superoxide anion production probably due to the action of intracellular RNS.

GAPDH is S-nitrosated during yeast apoptosis

Analysis of mitochondrial and total cellular proteome of H_2O_2-induced apoptotic cells revealed several GAPDH alterations, particularly of the Tdh3p isoenzyme (Fig. 6, Table 1). Upon H_2O_2 exposure, Tdh3p was detected within five spots of increased intensity, three of them corresponding to putative protein fragments, one to the mature protein with the putative mitochondrial import signal sequence removed (see Fig. S1 in supplementary material), and one corresponding to a new isoform of the complete Tdh3p with a lower isoelectric point. Tdh2p was also detected as a putative protein fragment (Fig. 6, Table 1; Fig. S1 in supplementary material), indicating that both GAPDH isoenzymes might be targets for proteolysis. In yeast, GAPDH, particularly the Tdh3p isoform, is normally found in the cytoplasm, the nuclei or the mitochondria depending on the physiological conditions (Ohlmeier et al., 2004). Interestingly, a new form of Tdh3p with a lower isoelectric point was detected in mitochondrial extracts (Fig. 6, Table 1), suggesting the occurrence of a posttranslational modification upon H_2O_2 treatment.

S-nitrosation promoted by NO has been shown to regulate GAPDH, a glycolytic protein extensively implicated in mammalian apoptosis (reviewed by Chuang et al., 2005). Besides NO production, our results revealed several different alterations of GAPDH upon H_2O_2-induced apoptosis, which prompted us to investigate whether GAPDH S-nitrosation and its involvement in apoptosis were probably conserved in yeast cells. The first approach explored the contribution of GAPDH isoenzymes (Tdh2p and Tdh3p) that, from the proteomic assay, were found to be altered during yeast cell death. Exposure of both *TDH2*- and *TDH3*-disrupted cells to apoptotic inducing

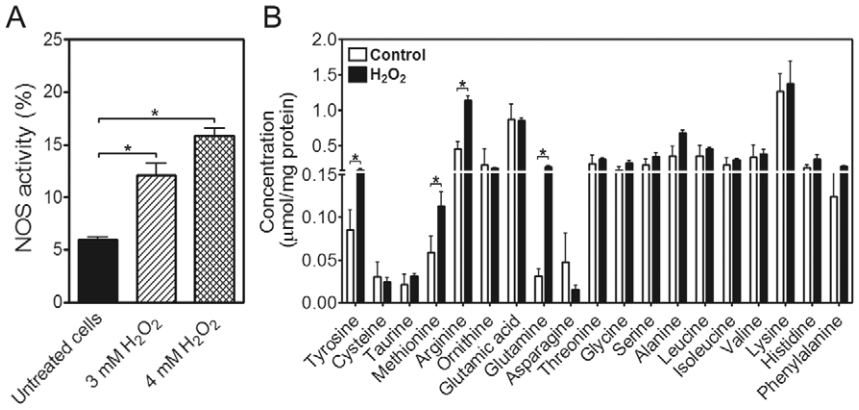

Fig. 3. H_2O_2-induced apoptotic cells display NOS-like activity. (A) NOS activity assessed in untreated and H_2O_2-treated wild-type cells. The radioactivity obtained from a negative control consisting of yeast extract boiled for 20 minutes was subtracted from all the samples to remove background radioactivity. Data are expressed as the percentage conversion of L-[^{3}H]arginine to [^{3}H]citrulline. *P≤0.03 versus untreated cells; t-test, n=4. (B) Intracellular amino acid concentrations of untreated (control) and H_2O_2-treated wild-type cells. *P≤0.05 versus untreated cells; t-test, n=3.

concentrations of H_2O_2 revealed an increase in the survival rate compared to that of wild-type cells (Fig. 7A), reflecting a putative role of GAPDH in the apoptotic process. Remarkably, Δ*tdh2* and Δ*tdh3* cells also displayed a reduction in intracellular ROS upon H_2O_2-induced apoptosis (Fig. 7B), suggesting the involvement of GAPDH in ROS generation during apoptotic cell death.

As a posttranslationally modified form of Tdh3p was detected in the mitochondria of H_2O_2-treated cells (Fig. 6, Table 1), we questioned if the concurrent synthesis of NO was promoting GAPDH S-nitrosation and its translocation to mitochondria. However, mass spectrometry analysis revealed that the observed mitochondrial GAPDH posttranslational modified form corresponds to an oxidation rather than an S-nitrosation of the protein (data not shown). Nevertheless, by immunoprecipitating GAPDH from cellular extracts with an anti-nitrosocysteine (CSNO) antibody, we demonstrated that GAPDH suffers a dose-dependent increase of S-nitrosation upon exposure to H_2O_2 (Fig. 8A,B). To support the observation of the occurrence of GAPDH S-nitrosation, cells were treated with the NO donor DETA/NO. The results showed that GAPDH also suffers S-nitrosation upon exposure to the NO donor, discarding a possible artefact of H_2O_2 treatment. Interestingly, treatment with H_2O_2 after pre-incubation with L-NAME resulted in a reduction in the amount of S-nitrosated GAPDH to control levels (Fig. 8C,D), associated with an increased survival rate of yeast cells (Fig. 4A). Furthermore, chronologically aged cells displayed increased GAPDH S-nitrosation (Fig. 8C,D), pointing to a role of NO and GAPDH in the signalling of yeast apoptosis.

In summary, the occurrence of GAPDH S-nitrosation reinforces the fact that during yeast apoptotic cell death, induced by H_2O_2 or age-associated, NO is produced, which is dependent on intracellular L-arginine content, and that, as in mammalian cells, it is responsible for the signalling and execution of the process through GAPDH action (Hara et al., 2005). These results show yeast to be a valuable model for

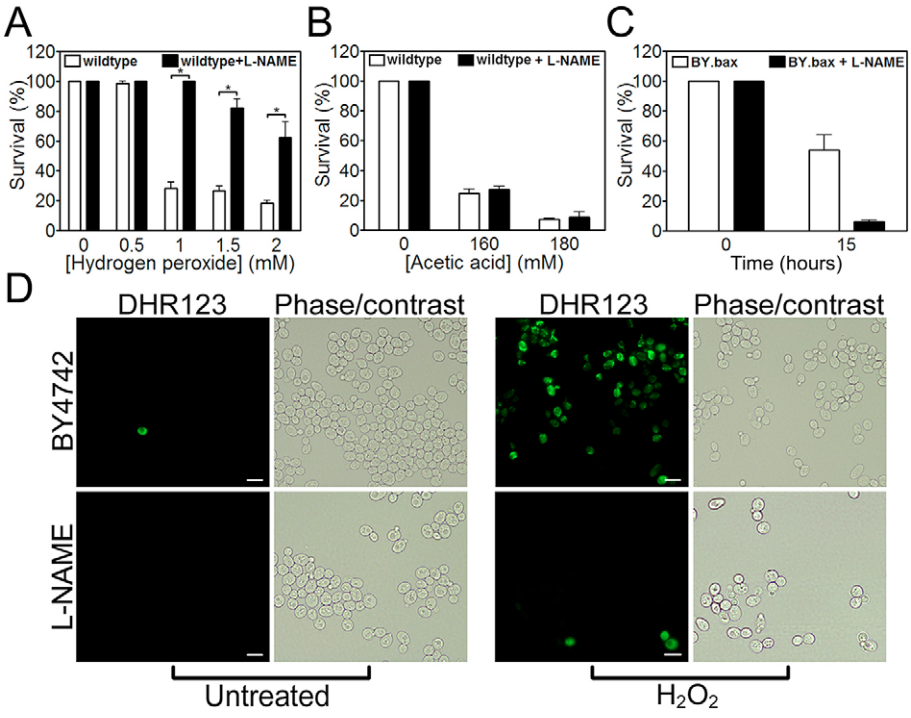

Fig. 4. Inhibition of NO production by L-NAME protects yeast cells from H_2O_2, but not from mammalian Bax expression, or acetic acid-induced apoptosis.
(A) Comparison of the survival rate of wild-type cells upon H_2O_2 treatment with or without pre-incubation with L-NAME in order to inhibit NO production. *P≤0.03 versus wild type; t-test, n=3.
(B) Comparison of the survival of wild-type cells upon acetic acid-induced apoptosis with or without pre-incubation with L-NAME. (C) Comparison of the survival of yeast cells (strain BY.bax) upon Bax expression for 15 hours (apoptotic inducing conditions), with or without pre-incubation with L-NAME.
(D) Epifluorescence and phase-contrast micrographs of untreated and H_2O_2-treated (1.5 mM) wild-type cells, with or without pre-incubation with L-NAME, stained with dihydrorhodamine 123 (DHR123) as an indicator of high intracellular ROS accumulation. Bars, 5 μm.

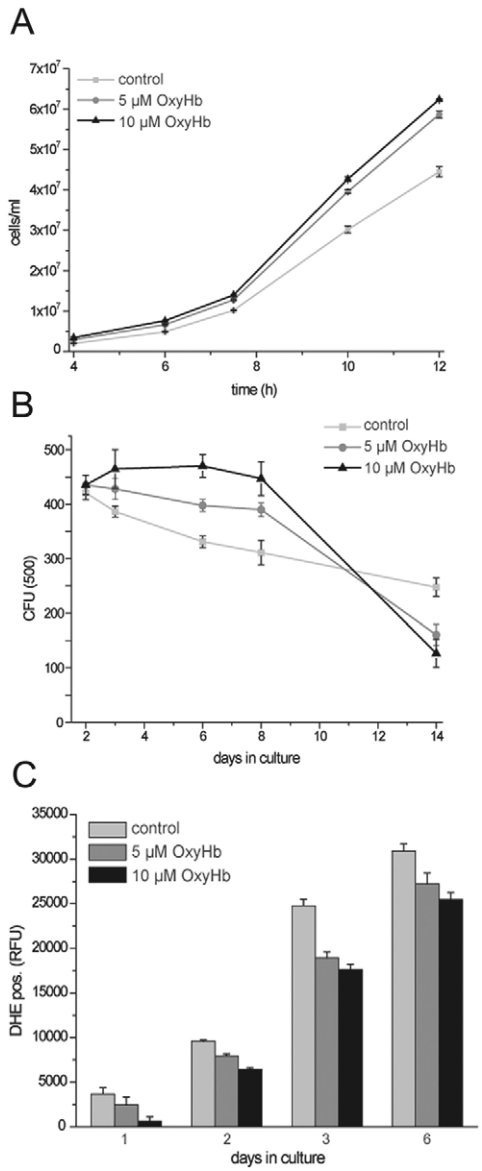

Fig. 5. NO scavenged by OxyHb is associated with a delay in cell death during chronological life span and to decreased levels of superoxide anion. (A) Growth curve of wild-type cells after addition of indicated concentrations of OxyHb. (B) Survival determined by clonogenicity during chronological aging of wild-type cells with or without addition of OxyHb, at the indicated concentrations on day 0. (C) Quantification (fluorescence) of ROS accumulation using dihydroethidium (DHE) staining during chronological aging of wild-type cells with or without OxyHb treatment.

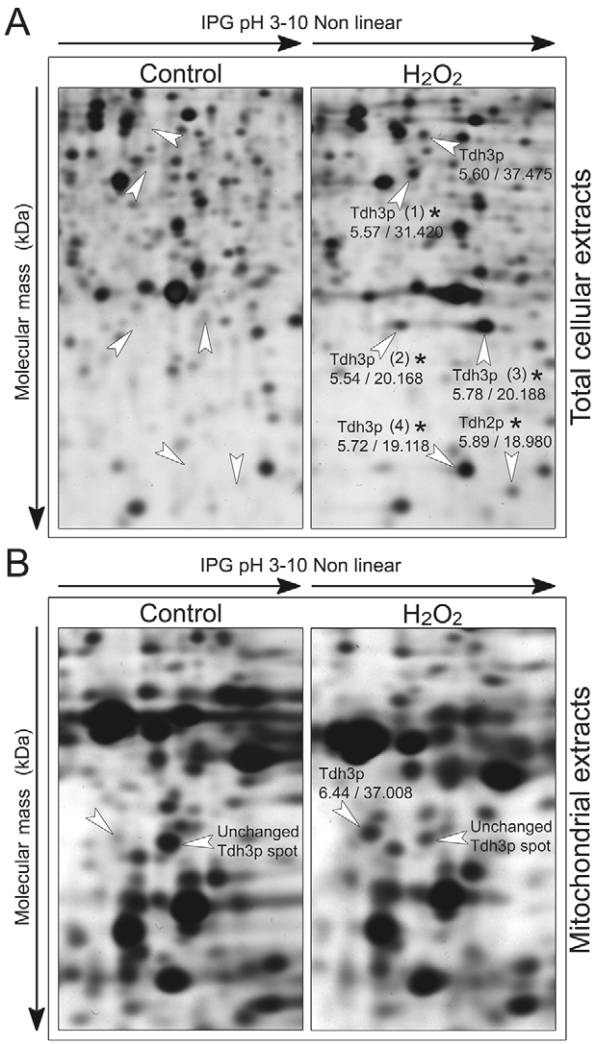

Fig. 6. GAPDH is extensively fragmented in H_2O_2-induced apoptotic cells. Comparison of protein expression levels in total cellular (A) and purified mitochondrial extracts (B) of untreated (control) and H_2O_2-treated wild-type cells. Selected regions of the 2-D gel (isoelectric point/molecular mass) are shown enlarged and the position of altered protein spots are marked with an arrowhead. The apparent isoelectric points and molecular masses of the proteins were calculated with Melanie 3.0 (GeneBio) using identified proteins with known parameters as a reference. Putative protein fragments are marked with an asterisk. Tdh3p fragments are numbered 1 to 4. For each Tdh3p and Tdh2p fragment, matched peptides obtained after trypsin digestion and used for identification of the proteins, as well as the amino acids specific for Tdh3p and Tdh2p, are shown in Fig. S1 in supplementary material.

studying the role of GAPDH in apoptosis and also open new frontiers for the study of NO role in yeast physiology.

Discussion

Apoptosis can be triggered by several different signals through different sub-programs controlled by a complex network of regulators and effectors. A large fraction of these apoptotic events depends on newly synthesized proteins, posttranslational modifications and translocation to specific cellular compartments (Ferri and Kroemer, 2001; Porter, 1999; Thiede and Rudel, 2004). Taking this into consideration, the analysis of cellular proteome under apoptotic conditions might produce useful information for the identification of apoptotic regulators and effectors. In our work, we examined the total and mitochondrial proteome of H_2O_2-induced apoptotic yeast cells. Proteomic analysis revealed the activation of stress-induced pathways through the increased levels of proteins previously described to be involved in both oxidative and nitrosative stresses. In addition, different posttranslationally modified forms of GAPDH were shown to be present at

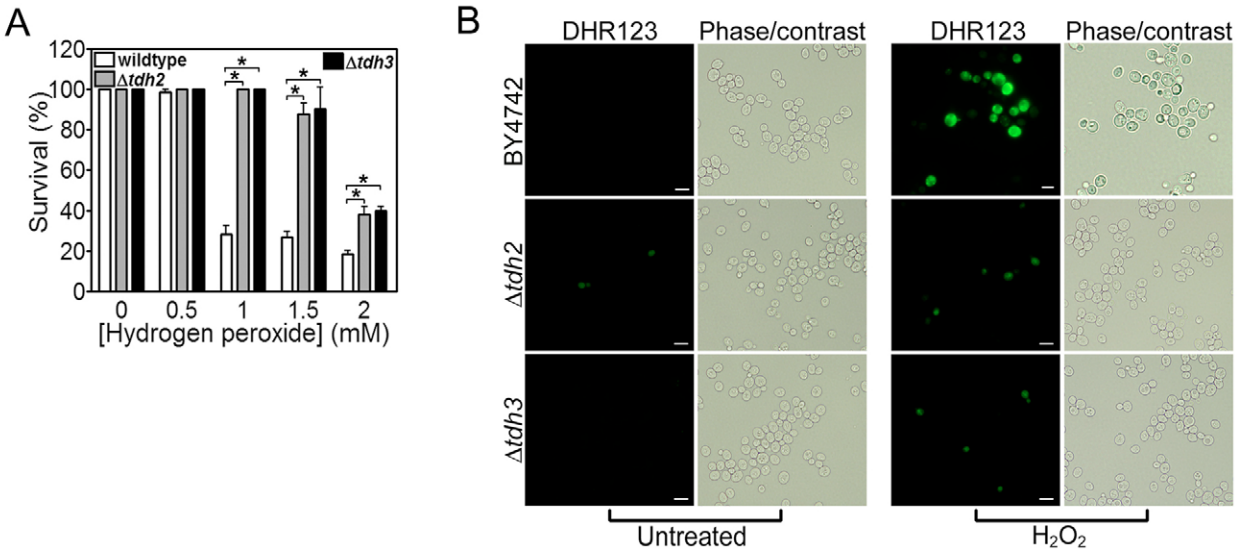

Fig. 7. Deletion of GAPDH isoform 2 and 3 prevents H_2O_2-induced apoptosis. (A) Comparison of the survival of wild-type, Δ*tdh2* and Δ*tdh3* cells upon H_2O_2 treatment, *$P \leqslant 0.03$ versus wild type, *t*-test, $n=3$. (B) Epifluorescence and phase-contrast micrographs of untreated and H_2O_2-treated (1.5 mM) wild-type, Δ*tdh2* and Δ*tdh3* cells, stained with dihydrorhodamine 123 (DHR123) as an indicator of high intracellular ROS accumulation. Bar, 5 μm.

increased levels, indicating that the activation of oxidative and nitrosative stress-induced pathways might have led to increased protein modifications, culminating in apoptotic cell death.

The observation of a nitrosative stress response, together with previous reports on endogenous NO production in yeast (Osorio et al., 2007), prompted us to examine the possibility of NO synthesis under H_2O_2 treatment. Our results show that H_2O_2 induces nitrosative stress as demonstrated by the indirect (measurement of nitrate concentration) and direct (NO-selective electrode and a NO-sensitive probe) detection of elevated intracellular NO levels, as well as by the detection of a NOS-like activity (classical methods used for mammalian cells). NO synthesis was found to be dependent on L-arginine and could be inhibited by the non-metabolized L-arginine analogous, L-NAME. The endogenous NO synthesis during H_2O_2-induced apoptosis, herein observed in yeast cells, has been previously described in mammalian cells, in which H_2O_2 activates endothelial NOS (Thomas et al., 2002), pointing to the conservation of some basic biochemical pathways activated/affected by H_2O_2. It is also clear that NO levels are mediating the apoptotic cell death occurring during chronological life span. Given that a common feature of apoptotic cell death induced by H_2O_2 or age-associated cell death is the generation of ROS, and bearing in mind that menadione was found to promote endogenous NO synthesis (Osorio et al., 2007), the relevance of NO in yeast apoptotic programs is reinforced, pointing to the conservation of links between ROS and RNS (Espey et al., 2000). Nevertheless, the origin of NO in yeast cells is still unclear, mainly due to the lack of mammalian NOS orthologues in the yeast genome. Although Castello and coworkers (Castello et al., 2006) showed that yeast cell mitochondria are capable of NO synthesis independently of a NOS-like activity, our results,

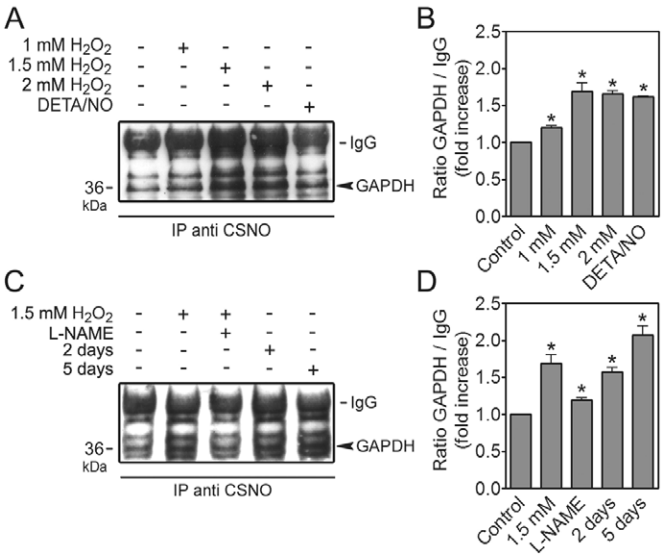

Fig. 8. GAPDH is S-nitrosated during H_2O_2-induced apoptosis. (A) Detection of S-nitrosated GAPDH by immunoprecipitation using an anti-CSNO antibody in cell extracts from untreated cells, H_2O_2-treated (1.0, 1.5 and 2.0 mM) cells and cells treated for 200 minutes with 2 mM of the NO donor diethylenetriamine/NO (DETA/NO). (B) Quantification of band intensity from A by densitometry. Band intensities were normalized to the intensity of IgG bands. Data express the GAPDH/IgG fold change in comparison to control (lane 1). *$P \leqslant 0.05$ versus control, *t*-test, $n=3$. (C) Immunoprecipitation of S-nitrosated GAPDH with an anti-CSNO antibody from cellular extracts of untreated, H_2O_2-treated (1.5 mM), either in the absence or presence of L-NAME, or chronologically aged cultures (2 and 5 days). (D) Quantification of band intensity from C by densitometry. Band intensities were normalized to the intensity of IgG bands. Data express the GAPDH/IgG fold change in comparison to control (lane 1). *$P \leqslant 0.05$ versus control, *t*-test, $n=3$.

together with the physiological role of NO, point to the presence of a yet unknown protein(s) with NOS-like activity in yeast. Plants, like yeast, do not have a protein with sequence similarity to known mammalian-type NOS but display a NOS-like activity, indicating the presence of an enzyme structurally unrelated to those of their mammalian counterparts.

Diverse cellular functions can be affected by NO through posttranslational modification, particularly S-nitrosation of GAPDH, a key glycolytic enzyme that undergoes S-nitrosation and translocates to the nucleus, triggering apoptosis in mammalian cells (Hara et al., 2005). Our results show that following H_2O_2 stimulus, yeast GAPDH is a target of extensive proteolysis as revealed by the number of identified fragments. Further studies concerning the elucidation of the functional relationship of GAPDH fragmentation with its apoptotic role will be crucial for the understanding of the evolutionarily conserved multifunction of GAPDH.

GAPDH has been previously described to suffer different posttranslational modifications upon an oxidative stress insult, both as a target (Magherini et al., 2007) and as the most abundant yeast S-thiolated protein in response to H_2O_2 challenge (Shenton and Grant, 2003). Surprisingly, only the Tdh3p and not the Tdh2p GAPDH isoenzyme is modified (Grant et al., 1999). In addition, GAPDH Tdh3p isoenzyme was also described as suffering a redox-dependent and reversible S-glutathiolation (reviewed by Klatt and Lamas, 2000), with the formation of proteins with different mixed disulfides probably encompassing NO-dependent S-nitrosation of protein thiol groups. In this study we show that H_2O_2 or the NO donor DETA/NO lead to GAPDH S-nitrosation, revealing that yeast GADPH is both S-nitrosated and S-glutathionylated as described for mammalian cells (Giustarini et al., 2005). This evidenced interrelationship between S-glutathiolation, thiol oxidation and nitrosation points to the formation of proteins with different mixed disulfides as a mechanism that integrates signalling by both oxidative and nitrosative stimuli (reviewed by Klatt and Lamas, 2000). Our results concerning NO synthesis, GAPDH fragmentation and S-nitrosation, together with the fact that ROS/RNS-induced S-glutathiolation is involved in the modulation of signal transduction pathways such as the regulation of proteolytic processing, ubiquitination and degradation of proteins (Klatt and Lamas, 2000), pinpoint yeast as an attractive model to uncover the emerging roles for ROS/RNS. Moreover, the role of GAPDH S-nitrosation seems to be of extreme importance for the yeast apoptotic process, since the blockage of GAPDH S-nitrosation by L-NAME is associated with decreased amounts of ROS within the cells, suggesting that S-nitrosated GAPDH also concurs with ROS generation, although by a mechanism that is still elusive, as it is in mammalian cells (Puttonen et al., 2006).

Altogether, our findings bring new insights into the evolutionarily conserved apoptotic pathways. Similarly to higher eukaryotes, yeast cells undergo apoptosis mediated by NO signalling, which places yeast as a powerful tool in the study of the mechanisms that determine cellular sensitivity to NO and for the elucidation of NO pro- and anti-apoptotic functions. Moreover, the finding that S-nitrosated GAPDH is involved in yeast apoptosis raises the possibility of future investigations using yeast cells to screen for drugs that directly act against S-nitrosated GAPDH.

Materials and Methods

Strains, media and treatments

S. cerevisiae strain BY4742 (*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) and the respective knockouts in the *TDH2* and *TDH3* genes (EUROSCARF, Frankfurt, Germany) were used. For H_2O_2 treatment, yeast cells were grown until the early stationary growth phase in liquid YPD medium containing glucose (2%, w/v), yeast extract (0.5%, w/v) and peptone (1%, w/v). Cells were then harvested and suspended (10^7 cells/ml) in fresh YPD medium followed by the addition of 0.5, 1.0, 1.5 and 2.0 mM H_2O_2 and incubation for 200 minutes at 26°C with stirring (150 r.p.m.), as previously described (Madeo et al., 1999). After treatment, 300 cells were spread on YPD agar plates and viability was determined by counting colony-forming units (c.f.u.) after 2 days of incubation at 26°C. For proteomic analysis, experiments were performed in YPD medium and an equitoxic dose of H_2O_2 was used which induced 50% of apoptotic cell death, as evaluated by TUNEL assay after 200 minutes (data not shown). For determination of NOS activity and kinetic measurement of NO production with the NO-selective electrode, cells were treated for a shorter period using higher H_2O_2 concentrations (3 and 4 mM) in order to induce 50% of apoptotic cell death. For acetic acid treatment, yeast cells were grown until the early stationary growth phase as described previously (Ludovico et al., 2002), harvested and suspended in YPD medium (pH 3.0 set with HCl) containing 0, 160 and 180 mM of acetic acid. Treatments were carried out for 200 minutes at 26°C. Viability was determined by c.f.u. counts as described above.

For determination of chronological life span and growth rates, cells were grown on synthetic complete (SC) medium containing glucose (2%, w/v), yeast nitrogen base (Difco) (0.17%, w/v), $(NH_4)_2SO_4$ (0.5%, w/v) and 30 mg/l of all amino acids (except 80 mg/l histidine and 200 mg/l leucine), 30 mg/l adenine and 320 mg/l uracil. For chronological aging experiments, cells were inoculated to an OD_{600}=0.1, oxyhaemoglobin (OxyHb) was added at the indicated concentrations (day 0) and viability was determined by counting c.f.u. For determination of proliferation rates, cells were inoculated to 5×10^5 cells/ml, OxyHb was added and cell number was determined using a CASY cell counter.

Bax expression was induced as described before (Madeo et al., 1999). In brief, strain BY4742 was transformed with plasmid pSD10.a-Bax (Madeo et al., 1999), which contains murine bax under the control of a hybrid GAL1-10/CYC1 promoter; originating strain BY.bax. Individual clones were pre-grown overnight in SC medium with glucose (2%, w/v) until exponential growth phase. To induce bax expression, cells were washed three times with water and resuspended in SC medium with galactose (2%, w/v). Cells were then incubated at 26°C with stirring (150 r.p.m.) for 15 hours. Viability was determined by c.f.u. counts as described above.

2-D gel electrophoresis

For analysis of total cell extracts, cells were collected and washed twice with 2 ml TE buffer (1 mM EDTA, 0.1 M Tris-HCl pH 7.5, complete mini protease inhibitor; Roche Applied Science, Mannheim, Germany). Cells were disrupted using a French Press with 900 p.s.i. (62.1 bar) and the cell lysate centrifuged. For analysis of mitochondrial extracts, cells were collected and mitochondria isolated and purified as previously described (Meisinger et al., 2000). Protein concentrations were determined with a commercially available kit (RotiNanoquant, C. Roth, Karlsruhe, Germany) and protein aliquots of total cell extracts, as well as mitochondrial extracts, (100 μg, 600 μg) were stored at –20°C. For two dimensional (2-D) gel electrophoresis the protein pellet was resuspended in urea buffer [8 M urea, 2 M thiourea, 1% (w/v) CHAPS, 20 mM 1,4-dithio-DL-threitol, 0.8% (v/v) carrier ampholytes], and complete mini protease inhibitor. The protein separation was done as previously described (Gorg et al., 1995). Briefly, the protein solution was adjusted with urea buffer to a final volume of 350 μl and in-gel rehydration performed overnight. Isoelectric focusing was carried out in IPG strips (pH 3-10, non linear, 18 cm; Amersham Biosciences, Uppsala, Sweden) with the Multiphor II system (Amersham Biosciences) under paraffin oil for 55 kVh. SDS-PAGE was done overnight in 12.5% T, 2.6% C polyacrylamide gels using the Ettan DALT II system (Amersham Biosciences) at 1-2 W per gel and 12°C. The gels were silver stained and analyzed with the 2-D PAGE image analysis software Melanie 3.0 (GeneBio, Geneva, Switzerland). The apparent isoelectric points (pI) and molecular masses (in kDa) of the proteins were calculated with Melanie 3.0 (GeneBio) using identified proteins with known parameters as a reference. An expression change was considered significant if the intensity of the corresponding spot reproducibly differed more than threefold.

Identification of altered proteins by mass spectrometry

Excised spots were in-gel digested and identified from the peptide fingerprints as described elsewhere (Gorg et al., 1995). Proteins were identified with the ProFound database, version 2005.02.14 (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) using the parameters: 20 ppm; 1 missed cut; MH+; +C2H_2O_2@C (Complete), +O@M (Partial). The identification of a protein was accepted if the peptides (mass tolerance 20 ppm) covered at least 30% of the complete sequence. Sequence coverage between 30% and 20% or sequence coverage below 20% for protein fragments was only accepted if at least two main peaks of the mass spectrum matched with the sequence and the number of weak-intensity peaks was clearly

reduced. The spot-specific peptides in the mass spectrum were also analyzed to confirm which parts of the corresponding protein sequence matched with these peptides, indicating putative fragmentation. This comparison reveals that spots presenting putative fragments lacked peptides observed in the mass spectrum of the whole protein. Thus, the spot position observed by 2-D gel electrophoresis and the specific peptides in the corresponding mass spectrum were analyzed to define the spot as intact protein or putative fragment. Distinction between GAPDH isoform 2 and 3 (Tdh2p and Tdh3p) was possible by the identification of amino acids present in the matched peptides that are specific for each GAPDH isoform.

Epifluorescence microscopy and flow cytometry analysis

Images were acquired using an Olympus BX61 microscope equipped with a high-resolution DP70 digital camera and using an Olympus PlanApo 60× oil objective, with a numerical aperture of 1.42. All the samples were suspended in PBS and visualized at room temperature.

Flow cytometry assays were performed on an EPICS XL-MCL flow cytometer (Beckman-Coulter Corporation, USA), equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Twenty thousand cells per sample were analyzed. The data were analyzed with the Multigraph software included in the system II acquisition software for the EPICS XL/XL-MCL version 1.0.

Assessment of intracellular reactive oxygen species (ROS)

Free intracellular ROS were detected with dihydrorhodamine 123 (DHR123) (Molecular Probes, Eugene, OR, USA). DHR123 was added from a 1 mg/ml stock solution in ethanol, to 5×10^6 cells/ml suspended in PBS, reaching a final concentration of 15 μg/ml. Cells were incubated for 90 minutes at 30°C in the dark, washed in PBS and visualized by epifluorescence microscopy. For dihydroethidium (DHE) staining, 5×10^6 cells were harvested by centrifugation, resuspended in 250 μl of 2.5 μg/ml DHE in PBS and incubated in the dark for 5 minutes. Relative fluorescence units (RFU) were determined using a fluorescence reader (Tecan, GeniusPRO™).

Indirect assessment of NO levels through nitrate concentration measurement

Nitrite and nitrate concentration was measured spectrophotometrically using the Griess-reagent. Sodium nitrite (0, 1.0, 2.0, 3.0, 5.0, 10, 15, 20 μM) was used as standard. For the reagent, 20 mg *N*-1-naphthylethylenediamine dihydrochloride, 200 mg suflanilamide and 2.8 g HCl (36%) was dissolved in 17.2 g water. Individual supernatant samples (100 μl) were mixed with 100 μl reagent and the concentration recorded. For nitrate concentration, 100 μl of vanadium (III) chloride (8 mg/ml 1 M HCl) was added, thus reducing any existent nitrate to nitrite. After incubation (90 minutes at 37°C) the concentration was recorded again. Nitrate concentration was calculated as the difference between the two measurements. Since NO is a diffusible free radical rapidly oxidized to nitrate and nitrite, the nitrate concentration obtained was assumed to be correlated to the amount of NO synthesized by the cells.

Direct assessment of NO levels

Intracellular NO levels upon H_2O_2 treatment, with or without the inhibition of NO production by the non-metabolized L-arginine analogue, N_ω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) were assessed by flow cytometry using the NO-sensitive probe 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM) diacetate (Molecular Probes, Eugene, OR, USA). After treatment, 3×10^7 cells were harvested, washed and suspended in PBS, pH 7.4. Cells were then incubated for 30 minutes at room temperature, with DAF-FM diacetate (5 μM).

NO production was kinetically measured using the AmiNO-700 Nitric Oxide Sensor with inNO Model-T – Nitric Oxide Measuring System (Innovative Instruments, Inc., Florida, USA). This NO electrode is specific to NO and has the detection limit of 0.1 nM, which is 20 times more sensitive than that of the ISO-NO electrode (World Precision Instruments, Florida, USA). For NO measurement, 5×10^8 cells (with or without pre-incubation with D-arginine or L-NAME, to inhibit NO production) were washed, resuspended in 3 ml of Tris buffer (10 mM Tris-HCl, pH 7.4) and transferred to a recording cell chamber with agitation, under aerobic conditions, followed by addition of 4 mM H_2O_2. A negative control consisting of Tris buffer without cells was also included to exclude possible H_2O_2 interferences with the electrode. Amperometric currents originated from the oxidation of NO at the electrode surface were recorded at +0.9 V. The electrode was calibrated in 100 mM KI-H_2SO_4 with stock solutions of nitrite according to the manufacturer's instructions.

Inhibition of NO production

For inhibition of NO production, cells were pre-incubated, for 1 hour, with L-NAME (200 mM) in YPD medium or pre-incubated for 1 hour with 0.4 mg/ml D-arginine. A high concentration of L-NAME was used throughout the work because of the presence of a cell wall in yeast cells.

Determination of NOS activity

The conversion of L-[^{3}H]arginine to [^{3}H]citrulline (NOS activity) was monitored using a highly sensitive Nitric Oxide Synthase Assay Kit (Calbiochem) with minor changes from the supplied protocol. Untreated or H_2O_2-treated yeast cells were harvested, washed in double-distilled water and resuspended in 25 mM Tris-HCl, pH 7.4; 1 mM EDTA and 1 mM EGTA. Yeast protein extracts were obtained by vortexing in the presence of 1 g of glass beads and used immediately after. 10 μl of protein extract (2 μg/μl) were added to 40 μl of reaction buffer with 1 μCi of L[^{3}H]arginine (60 Ci/mmol), 6 μM tetrahydrobiopterin, 2 μM FAD, 2 μM FMN, 1 mM NADPH, 0.6 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.4. After 60 minutes of incubation, 400 μl of EDTA buffer (50 mM Hepes pH 5.5, 5 mM EDTA) were added. In order to separate L-arginine from citrulline, 100 μl of equilibrated L-arginine-binding resin was added and samples were applied to spin cup columns and centrifuged. Citrulline quantification was performed by liquid scintillation spectroscopy of the flow-through. The radioactivity obtained from a negative control consisting of yeast extract boiled for 20 minutes was subtracted from all the samples to remove background radioactivity. Data are expressed as the percentage of conversion of L-[^{3}H]arginine to [^{3}H]citrulline and represent the average of four independent experiments.

Quantification of intracellular amino acids

For the intracellular amino acid quantification, untreated and H_2O_2-treated cells were disrupted as described above. Proteins were removed from the samples by TCA precipitation followed by sulfosalicylic acid clean-up and filtration. Samples were then analyzed by ion exchange column chromatography followed by post-column ninhydrin derivatization on an automated amino acid analyzer (Biochrome 30, Amersham Pharmacia Biotech, Cambridge, UK).

Detection of S-nitrosated GAPDH

S-nitrosated GAPDH was detected by immunoprecipitation with an anti-nitrosocysteine (CSNO) antibody. Briefly, untreated, H_2O_2-treated with or without pre-incubation with L-NAME, and aged cells were disrupted using glass beads as previously described (Gourlay et al., 2003). As a positive control, cellular nitrosative stress was induced by the NO donor (Cahuana et al., 2004; Horan et al., 2006) diethylenetriamine/NO (DETA/NO, Sigma-Aldrich). The treatment with a NO donor facilitated the increase of intracellular NO levels allowing the determination of GAPDH S-nitrosation independent of H_2O_2. Thus, cells were incubated for 200 minutes with 2 mM of DETA/NO. One mg of cell lysate was mixed with rabbit anti-S-nitrosocysteine antibody (Sigma-Aldrich) at a dilution of 1:160 and incubated at 4°C with rotation for 4 hours. Protein G plus/protein A-agarose beads were added and rotated overnight at 4°C. Immunoprecipitated proteins were then resolved on a 10% SDS gel and transferred to a PVDF membrane before being probed with a monoclonal mouse anti-GAPDH antibody (MAB474, Chemicon) at a dilution of 1:200. A horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was used (Chemicon) at a dilution of 1:5000 and detected by enhanced chemiluminescence.

Statistical analysis

The arithmetic means are given with standard deviation with 95% confidence value. Statistical analyses were carried out using independent samples *t*-test analysis. A *P* value less than 0.05 was considered to denote a significant difference.

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