RESEARCH ARTICLE

Metabolic response to MMS-mediated DNA damage in Saccharomyces cerevisiae is dependent on the glucose concentration in the medium

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Abstract
Maintenance and adaptation of energy metabolism could play an important role in the cellular ability to respond to DNA damage. A large number of studies suggest that the sensitivity of cells to oxidants and oxidative stress depends on the activity of cellular metabolism and is dependent on the glucose concentration. In fact, yeast cells that utilize fermentative carbon sources and hence rely mainly on glycolysis for energy appear to be more sensitive to oxidative stress. Here we show that treatment of the yeast Saccharomyces cerevisiae growing on a glucose-rich medium with the DNA alkylating agent methyl methanesulphonate (MMS) triggers a rapid inhibition of respiration and enhances reactive oxygen species (ROS) production, which is accompanied by a strong suppression of glycolysis. Further, diminished activity of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase upon MMS treatment leads to a diversion of glucose carbon to glycerol, trehalose and glycogen accumulation and an increased flux through the pentose-phosphate pathway. Such conditions finally result in a significant decline in the ATP level and energy charge. These effects are dependent on the glucose concentration in the medium. Our results clearly demonstrate that calorie restriction reduces MMS toxicity through increased respiration and reduced ROS accumulation, enhancing the survival and recovery of cells.

Introduction
Modulation in energy metabolism seems to play an important role in the cellular response to DNA damage. Gene expression studies of cells exposed to various stress conditions including DNA damage showed, in addition to other gene expression changes, significant regulation of genes involved in glycolysis and gluconeogenesis (DeRisi et al., 1997; Godon et al., 1998; Dumond et al., 2000; Jelinsky et al., 2000; Causton et al., 2001; Gasch et al., 2001). A potential effect of these changes in glucose metabolism could be a shift from glycolysis to the pentose-phosphate pathway (PPP), as suggested by Dumond et al. (2000), which could lead to the generation of reducing equivalents (NADH and NADPH) required for cellular antioxidant systems. That oxidative stress response is also crucial in the metabolic response to DNA damage became evident based on the observation that DNA damage mediated by methyl methanesulphonate (MMS) induces the formation of reactive oxygen species (ROS) in yeast (Salmon et al., 2004; Kitanovic & Wölf, 2006). Moreover, suppression of ROS formation due to altered (glucose) metabolism as well as ROS scavenging improves the survival of MMS-treated cells (Kitanovic & Wölf, 2006). The diauxic shift represents a physiological condition that contributes to increased oxidative-stress tolerance of stationary-phase yeast cells (Jamieson et al., 1994; Steels et al., 1994). In addition, transcription of many antioxidant defence genes is repressed by glucose and their derepression usually follows glucose exhaustion (Krems et al., 1995).

Glycolytic enzymes were reported to be the target of oxidative modifications (Albina et al., 1999; Arutyunova et al., 2003; Osório et al., 2003, 2004; Shenton & Grant, 2003; Yoo & Regnier, 2004; Almeida et al., 2007). The toxic effect of oxidants or alkylating agents appeared to be
dependent on the glucose concentration and the cellular metabolic state (Osório et al., 2004; Zong et al., 2004; Lopez et al., 2005). Bearing in mind that cancer cells are strongly dependent on glycolysis and that they generally exhibit a poor antioxidant status (reviewed in Verrax et al., 2008), detailed investigations of the metabolic response and its alternations upon treatment with cytotoxic and genotoxic substances could be of considerable interest for future anticancer therapy.

Despite this interconnection between energy metabolism and sensitivity to toxic stress in various cell types, no detailed analysis of the metabolic flux in response to a DNA-damaging agent has been presented so far. MMS is a highly toxic DNA-alkylating agent that methylates DNA at 7-deoxyguanine and 3-deoxyadenine, causing base mispairing and replication blocks that activate DNA damage repair pathways and cell cycle arrest (Evensen & Seeberg, 1982). There are ample evidences indicating that upon treatment with alkylating agents such as MMS or N-methyl-N-nitrosourea, mtDNA is preferentially alkylated in relation to nuclear DNA (Wunderlich et al., 1970; LeDoux et al., 1992; Pirsel & Bohr, 1993). It is also known that DNA alklylation lesions trigger the mitochondrial apoptotic pathway (Kaina et al., 1997; Tominaga et al., 1997; Meikrantz et al., 1998). Because of this ability, DNA-alkylating agents have been used for many years in cancer research and in the treatment of several human cancers (Beranek, 1990), which is, however, hampered by sytemic toxicity and drug resistance, limiting their clinical efficacy (Ralhan & Kaur, 2007).

Here we present a detailed analysis of the physiological and metabolic alterations in yeast cells upon treatment with MMS. MMS treatment led to a strong suppression of the enzymatic activity of two major glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK), and to an inhibition of oxygen consumption. The latter effect was strongly dependent on the glucose concentrations in the medium. Stimulation of mitochondrial activity before MMS treatment significantly reduced oxidative damage in the cells and improved survival. Altogether, the cellular energy state appeared to be an important factor in the cellular response to MMS treatment.

Materials and methods

Strains, media and growth conditions

The yeast strains used in this study are: FF 18984 (MATa leu2-3,112 ura3-52, lys2-1, his7-1, Δhap4 (MATa leu2-3,112 ura3-52, lys2-1, his7-1::Hap4::KanMX4)). Yeast cells were grown in rich (YPD) medium containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ Bacto peptone and different glucose concentrations (as indicated; 20, 10, 5 or 2.5 g L⁻¹). YPKG contained 10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto peptone, 10 g L⁻¹ potassium acetate and 5 g L⁻¹ glucose. Cell growth was followed by optical absorbance readings at 600 nm. For treatment with MMS, medium was inoculated with overnight precultures and grown at 30 °C to the mid-log phase (OD₆₀₀ nm 0.6–0.8). Cultures were divided and either mock-treated or treated with indicated concentrations of MMS. For dry-weight measurements, polyamide filters (pore size 0.45 μm; Sartorius, UK) were used. After removal of the medium by filtration, the filter was washed with demineralized water and dried to a constant weight in a glass beaker exicator under vacuum.

Metabolic byproduct determination and glucose consumption assay

In order to quantify glucose consumption and metabolic byproduct accumulation, the culture supernatant was collected at the indicated time points. Glucose, glycerol, ethanol and acetate were determined by HPLC. The cell-free supernatant was filtered through 0.22-μm pore-size nylon filters before loading on an HPX-87H Aminex ion exclusion column. The column was eluted at 48 °C with 5 mM H₂SO₄ at a flow rate of 0.5 mL min⁻¹ and the concentration of the compounds was determined using a Waters model 410 refractive index detector.

Quantitative assessment of glycogen and trehalose content

The procedure was performed as described previously (Parrou & François, 1997). Briefly, the cell pellet (collected from 20 OD₆₀₀ nm units of culture) was suspended in 250 μL 0.25 M Na₂CO₃ and heated at 95 °C for 4 h with occasional stirring. The suspension was adjusted to pH 5.2 with 150 μL 1 M acetic acid and 600 μL 0.2 M sodium acetate buffer, pH 5.2. Half of this mixture was incubated overnight at 57 °C with continuous shaking on a rotary shaker in the presence of 100 μg of α-amyloglucosidase from Aspergillus niger (Sigma). The second half of the mixture was incubated overnight at 37 °C in the presence of 3 mU trehalase (Sigma). The glucose released was determined using the glucose oxidase/peroxidase method (Cramp, 1967).

Metabolite extraction and analysis of nucleotide content

The sampling method was performed as described previously (Loret et al., 2007). Briefly, portions (20 OD₆₀₀ nm units) of the cell culture were rapidly collected by filtration, suspended in 5 mL buffered ethanol (75% ethanol in 10 mM HEPES, pH 7.1) and incubated for 4 min at 85 °C. The
solvent was evaporated to dryness at 37 °C under vacuum and the cell extract was suspended in 0.5 mL deionized water. The nucleotide content was analysed by high performance ionic chromatography (HPIC) carried out on a DX 500 chromatography workstation (Dionex, Sunnyvale, CA) equipped with a GP50 gradient pump, ED50 electrochemical and UV detectors as described in Loret et al. (2007).

**Energy charge**

Energy charge is defined in terms as the sum of hydrolysable phosphate bonds in adenine nucleotides over the total concentration of these nucleotides, calculated from the actual concentrations as ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]) (Atkinson, 1966).

**Preparation of cell-free extracts and enzyme assays**

All procedures were carried out at 0–4 °C. Crude extracts were prepared from 20 OD600 nm units of cells with 1-g glass beads (0.4–0.5 mm diameter) in 0.5 mL 20 mM HEPES, pH 7.1, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA and 1 mM dithiothreitol. Samples were vortexed (3 × 5 min with cooling on ice in between) in Mixer Mill MM 300 (Retsch). After centrifugation at 16 000 g for 15 min at 4 °C, the supernatants were immediately used for enzymatic assays. The protein content was determined using the method of Bradford (1976). All chemicals and enzymes for enzymatic assays were purchased from Sigma.

GAPDH (EC 1.2.1.12) activity was measured at 30 °C by coupling the production of glyceraldehyde-3-phosphate from 3-phosphoglyceric acid to the consumption of NADH, using a spectrophotometric assay in a coupled 3-phosphoglycerate phosphokinase–GAPDH system (Bergmeyer et al., 1974). The reaction was performed in 0.15 mL buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM KCl, 2.5 mM MgCl2, 0.16 mM acetyl-CoA, 1.67 mM NADH, 6.67 mM malic acid and 3 U of malate dehydrogenase (EC 1.1.1.37). The reaction was initiated by the addition of crude extract, and increase in A340 nm was monitored.

PYK (EC 2.7.1.40) activity was measured at 30 °C by coupling the production of pyruvate from phosphoenolpyruvate and ADP to the consumption of NADH, using a spectrophotometric assay in a coupled PYK–lactic dehydrogenase system (Bergmeyer et al., 1974). The reaction was performed in 0.15 mL buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM KCl, 2.5 mM MgCl2, 10 mM ATP/Mg, 0.7 mM NADH, 6.7 mM 3-phosphoglyceric acid and 10 U of 3-phosphoglyceric phosphokinase (EC 2.7.2.3). The reaction was initiated by the addition of crude extract and was followed by a decrease in A340 nm.

PYK (EC 2.7.1.40) activity was measured at 30 °C by coupling the production of pyruvate from phosphoenolpyruvate and ADP to the consumption of NADH, using a spectrophotometric assay in a coupled PYK–lactic dehydrogenase system (Bergmeyer et al., 1974). The reaction was performed in 0.15 mL buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM KCl, 2.5 mM MgCl2, 10 mM ATP/Mg, 0.7 mM NADH, 1.7 mM phosphoenolpyruvate and 10 U of lactic dehydrogenase (EC 1.1.1.27). The reaction was initiated by the addition of crude extract, and decrease in A340 nm was monitored.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured at 30 °C by measuring NADPH production, using a spectrophotometric assay (Noltmann et al., 1961). The reaction was performed in 0.15 mL buffer containing 50 mM imidazole, pH 7.1, 100 mM KCl, 5 mM MgSO4, 5 mM EDTA, 0.7 mM NADP+ and 3.4 mM glucose-6-phosphate. The reaction was initiated by the addition of crude extract, and increase in A340 nm was monitored.

Citrate synthase (CS; EC 2.3.3.1) activity was measured at 30 °C by coupling the production of oxalacetate and NADH from malic acid and NAD+ to the consumption of oxalacetate by CS, using a spectrophotometric assay in a coupled malic dehydrogenase–CS system (Srere, 1969). The reaction was performed in 0.15 mL buffer containing 50 mM Tris-HCl, pH 7.1, 5 mM KCl, 2.5 mM MgCl2, 0.16 mM acetyl-CoA, 1.67 mM NAD+, 6.67 mM malic acid and 3 U of malate dehydrogenase (EC 1.1.1.37). The reaction was initiated by the addition of crude extract, and increase in A340 nm was monitored.

**Toxicity assay**

Wild-type strain FF18984 was grown in YPD and YPKG media until the mid-log phase (OD600 nm = 0.6–0.8). OD600 nm was adjusted to 0.5 and five additional fivefold serial dilutions were made. Four microlitres of each serial dilution were spotted onto agar plates containing the indicated media and incubated at 30 °C for 3 days.

**Detection of ROS level, cardiolipin content (CL), mitochondrial activity and mitochondrial membrane potential (Ψm) by flow cytometry analysis**

Control and MMS-treated cells were grown until the mid-log or the stationary phase. An aliquot of cells was taken at the indicated time and cells were dissolved in phosphate-buffered saline (PBS). For the ROS level, samples were incubated for 10 min with dihydroethidium (Molecular Probes) at a final concentration (f.c.) of 5 μg mL−1. Cells were washed and suspended in PBS. ROS production was quantified using FACS® Calibur (Becton Dickinson) and CELLQuest PRO ANALYSIS software. Excitation and emission settings were 488 and 564–606 nm (FL2 filter). The CL was determined by 15-min staining with 10 nonyl acridine orange (f.c. 0.5 μM) (NAO; Molecular Probes). Cells were washed and fluorescence was quantified using FACS® Calibur (Becton Dickinson) and CELLQUEST PRO ANALYSIS software. Excitation and emission settings were 488 and 525–550 nm (FL1 filter). The mitochondrial membrane potential was analysed after 15 min of incubation with rhodamine 123 (f.c. 10 μM) (Molecular Probes; Chen et al., 1982). The excitation and emission settings were 488 and 564–606 nm (FL2 filter). Active mitochondria were quantified by 15 min Mitto Tracker Green FM labelling (f.c.
100 nM) (Molecular Probes). Excitation and emission settings were 488 and 564–606 nm (FL2 filter). Dead cells were excluded from the statistical analysis of CL and Mito Tracker Green intensity by parallel staining with propidium iodide (PI).

**Monitoring of oxygen consumption**

Oxygen consumption was monitored in OxoPlate® (Pre-Sens, Germany) covered with a breathable membrane (Diversified Biotech) in 150 μL volume containing the indicated medium and substances and 0.1 OD$_{600 nm}$ units of cells. The signal of the oxygen fluorescence sensor and the OD of the culture at 600 nm were measured continuously during the indicated time at kinetic intervals of 30 min. The calibration of the fluorescence reader was performed using a two-point calibration curve with oxygen-free water (80 mM Na$_2$SO$_3$) and air-saturated water. The partial pressure of oxygen was calculated from the calibration curve.

**Determination of cofactors**

The cellular levels of NADP$^+$ and NADPH were determined using the method of Klingenberg (1989). Cells from 24 h MMS-treated cells were divided into two parts, and acid (0.5 M perchloric acid) or alkaline extracts (0.5 M alcoholic potassium hydroxide solution) were prepared to measure NADP$^+$ and NADPH levels, respectively. The level of NADPH produced in an enzymatic reaction with G6PDH was analysed spectrophotometrically at 340 nm.

The cellular levels of NAD$^+$ and NADH were determined using the cycling method of Matsumura & Miyachi (1983). Cells from 24 h MMS-treated cells were divided into two parts, and acid (0.1 M hydrochloric acid) or alkaline extracts (0.1 M sodium hydroxide solution) were prepared to measure NAD$^+$ and NADH levels, respectively. The method involves coupling of a NAD$^+$-dependent (alcohol) dehydrogenase reaction with the reduction of tetrazolium salt. The reaction was performed in 0.15 mL buffer containing 87 mM triethanolamine hydrochloride, pH 7.4, 9.6% ethanol, 2 mM phenazine ethosulphate, 2 mM 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, 4 mM EDTA and 1.5 U of alcohol dehydrogenase. The reaction was initiated by the addition of acid or alkaline extracts, and increase in A$_{570 nm}$ was monitored.

**Mitochondria isolation and oxygen consumption of isolated mitochondria**

Mitochondria were isolated from the stationary culture of cells grown in full (YPD) medium. Cells were spheroplasted with zymolase and subsequently broken by glass beads in an ice-cold sorbitol buffer (0.6 M sorbitol, 20 mM HEPES, 1 mM EDTA and 0.2% bovine serum albumin, pH 7.4). Lysate was purified from unbroken cells and cell debris by centrifugation at 500 g for 5 min and mitochondria were pelleted by centrifugation at 10 000 g for 10 min. Mitochondria were suspended in ice-cold sucrose buffer (250 mM sucrose, 20 mM HEPES and 1 mM EDTA, pH 7.4). Mitochondrial activity was stimulated with 12.5/12.5 mM glutamate/malate and 1.65 mM ADP and oxygen consumption was measured in sorbitol buffer containing MMS (0.01%, 0.02% and 0.04%) or 3 mM H$_2$O$_2$ in OxoPlate®.

**Results**

**Bioenergetic conditions in yeast cells upon MMS treatment-growth rate and glucose consumption**

We discovered earlier that changes in the expression of the gluconeogenic protein, fructose-1,6-bisphosphatase, significantly modulate cellular sensitivity to the DNA-damaging agent MMS (Kitanovic & Wölf, 2006). To understand better the role of glucose metabolism and bioenergetic conditions in the cellular sensitivity to treatment with this alkylating agent, we analysed whether treatment with MMS alters carbon flux through the main metabolic pathways, glycolysis, tricarboxylic acid (TCA) and PPP. Wild-type strain FF18984 was cultivated in full medium (YPD; with 2% glucose), mid-log phase cultures were split and either mock- or MMS-treated. Filtrated medium from several time points during a 24 h time course was collected and the concentrations of extracellular metabolites, glucose, ethanol, glycerol and acetate were analysed by HPLC. Upon treatment with 0.03% MMS cellular growth is immediately affected, resulting in a reduced OD at all time points (Fig. 1a), which is consistent with a rapid cell cycle arrest in the S phase reported earlier (Gasch et al., 2001). After a 24 h treatment with 0.03% MMS, OD is reduced > 50%. The analysis of glucose consumption from the medium revealed that control cells grown in full medium consumed the entire glucose within 6 h. In contrast, in MMS-treated cultures, glucose consumption started to reduce after 4 h of treatment, but still the entire glucose was consumed after 24 h. This consumption occurred in the absence of growth as MMS strongly inhibited biomass production during this period (Fig. 1a).

Measurement of ethanol, glycerol and acetate production revealed that the maximal byproduct accumulation in control cultures is reached within 6 h of cultivation in full medium corresponding to the complete exhaustion of glucose (Fig. 1b–d). After this time point, the concentrations of ethanol, glycerol and acetate started to decrease over time, indicating a switch from fermentative to respiratory metabolism and utilization of nonfermentative carbon.
sources. In cultures treated with MMS, ethanol, glycerol and acetate were also produced along with glucose consumption. However, no decrease in the extracellular concentration of these byproducts was observed upon glucose exhaustion, indicating that MMS-treated cells were not able to switch from fermentative to respiratory metabolism. In contrast, MMS-treated cells produced more glycerol than nontreated ones (Fig. 1c).

When nutrients become exhausted, yeast cells accumulate the storage carbohydrates, trehalose and glycogen. The intracellular concentration of trehalose and glycogen was also linked to the survival of yeast cells under various stress conditions (Parrou et al., 1997; Hounsa et al., 1998). Analysing cellular glycogen stores, a high accumulation of glycogen upon MMS treatment in glucose-rich medium could be detected (Fig. 2a). Consistent with the inability of MMS-treated cells to utilize nonfermentative carbon products from glucose fermentation, these cells mobilized their glycogen stores when glucose was exhausted (Fig. 2a). A similar profile of rapid glycogen mobilization was reported by Enjalbert et al. (2000) in respiratory-deficient mutants. Nontreated cells showed a very low trehalose content when grown in full (YPD) medium. Concerning trehalose synthesis, nontreated cells started to accumulate high levels of trehalose after glucose was consumed. The trehalose level in these cells remained high over 48 h, reaching 12% of cell dry weight at 24 h of cultivation (Fig. 2b). In MMS-treated cells trehalose accumulation occurred more rapidly but to a much lower level. At later time points, MMS-treated cells mobilized this carbohydrate storage to continue growth. Therefore, our results clearly suggest that MMS treatment reduces glucose utilization and glycolytic flux while increasing glycerol, trehalose and glycogen production at an early point of cultivation in a glucose-rich medium. The rapid mobilization of stored glucose from glycogen and trehalose as MMS-treated cells ran out of an exogenous carbon source is consistent with the inability of MMS-treated cells to utilize nonfermentative carbon sources and hence must thrive on their endogenous carbon stores for energy maintenance (Enjalbert et al., 2000; Jules et al., 2008).
MMS effects on the activity of glycolysis enzymes and the PPP

The above results clearly show that MMS treatment changes the function of the main energetic pathways and the carbon flux into various end products. One explanation could be a direct effect of MMS on the activity of metabolic enzymes. To test this hypothesis, we analysed the enzymatic activity of some key enzymes of glycolysis, TCA and PPP: PYK; GAPDH (yeast TDH); CS; and G6PDH (Fig. 3). Activities of PYK and GAPDH, two important enzymes of the lower part of glycolysis, were strongly reduced already after 4 h of MMS treatment, regardless of the medium in which cells were cultivated. In cells grown in low-glucose/acetate (YPKG) medium, PYK and GAPDH activities were significantly lower even in the untreated control (Fig. 3a and b). These lower activities might be due to lower expression under gluconeogenic conditions, as reported earlier (Gasch et al., 2000), which is in agreement with an increased requirement for oxidative phosphorylation under low-glucose conditions (Pronk et al., 1996).

Considering that MMS, similar to peroxide, triggers the formation of ROS even at lower concentrations (Salmon et al., 2004; Kitanovic & Wollf, 2006), redirection of the metabolic flux may also reflect an adjustment of antioxidative defence in response to MMS treatment. It was reported earlier that treatment with 1.5 mM H$_2$O$_2$ results in GAPDH inhibition under both low- and high-glucose conditions (Osório et al., 2004). We observed that peroxide treatment not only inhibited GAPDH but also reduced PYK activity in vivo treating cells with 3 mM H$_2$O$_2$ (data not shown). Inhibition of the lower glycolytic pathway, namely the processes controlled by GAPDH and PYK, could significantly influence NADH regeneration and by this, increase the NAD$^+$/NADH ratio and redirect the carbon flux to glycerol. Our measurements of these important redox cofactors show a significant increase of NAD$^+$ upon MMS treatment (Fig. 4a). The increase in glycerol production shown above (Fig. 1c) is also consistent with the GAPDH and PYK inhibition observed.

Adjustment of the cellular redox potential is also observable in the PPP. While the activity of G6PDH was increased by about 30–50% when cells were treated for 4 and 24 h with MMS (Fig. 3c), we did not observe an increase of the NADPH/NADP$^+$ ratio (Fig. 4b). Although G6PDH is a major supplier of the reducing equivalent NADPH, fast recycling of NADPH in antioxidant defence reactions may explain the lack of a concomitant increase in the NADPH/NADP$^+$ ratio. This assumption is reflected in the reduced half-cell reduction potential upon MMS treatment. Interestingly, in cells grown in low-glucose/acetate medium (YPKG), we observed a significantly higher reduction potential due to an elevated level of NADPH (Fig. 4b). In YPKG medium, the reducing power is utilized upon MMS treatment, changing the NADPH/NADP$^+$ ratio despite the enhanced activity of G6PDH. This enhanced activity of G6PDH is probably an integral part of the antioxidant defence and is in accordance with early observations that enzymes of the PPP play an important role in protection from oxidative stress in both yeast and mammals (Pandolfi et al., 1995; Slekar et al., 1996).

Analysis of mitochondrial function through determination of CS activity revealed a slight increase of its activity at the beginning of treatment, and significantly decreased activity upon 24 h of incubation with MMS in full medium (Fig. 3d), indicating persistent glucose repression in MMS-treated cells. As expected, CS activity was elevated in respiratory active cells, for example cells from the YPD stationary phase (control culture upon 24 h cultivation) or those grown in YPKG medium, confirming glucose derepression and a switch from fermentative to oxidative.
metabolism. It is important to note that we did not detect a significant influence of MMS on CS activity in YPKG medium. This suggests that enhanced mitochondrial metabolism, reflected in enhanced CS activity, could serve as a protective mechanism against MMS, enabling more cells to survive and continue proliferation. Indeed, cells cultivated in YPKG survive MMS treatment much better, which is clearly observable in the plate sensitivity assay (Fig. 4c).

Inactivation of GAPDH and PYK activity by MMS as well as H$_2$O$_2$ (data not shown) raised the question of whether these enzymes were directly affected by MMS treatment or rather through subsequent ROS production and oxidative stress. Purified GAPDH and PYK or cell lysates were treated with different MMS and H$_2$O$_2$ concentrations for 2 h. While MMS did not significantly inhibit the activity of both enzymes, neither purified, nor in cellular lysate (Fig. 5), 3 mM H$_2$O$_2$ strongly inhibited GAPDH activity of the purified enzyme as well as in cell extracts by more than 80%. PYK activity was not influenced by H$_2$O$_2$. These results indicate that GAPDH inhibition upon MMS treatment is the result of high ROS production, while the effect of MMS or H$_2$O$_2$ on PYK activity in vivo is not a direct effect of ROS generated by these agents.

**Bioenergetic conditions in yeast cells upon MMS treatment – nucleotide levels and energy charge**

Our results showed that upon MMS treatment, all glucose is catabolized into byproducts, without concomitant growth. On the other hand, the activity of enzymes involved in dephosphorylation of C3 units is significantly decreased, suggesting reduced flux into pyruvate production. Changes in the glycolytic flux could significantly influence cellular energy homeostasis, which should affect the cellular antioxidative and DNA damage response, both high energy-demanding processes. To address this question, we measured the levels of nucleotide metabolites and estimated the energy status of MMS-treated cells. MMS treatment significantly reduced ATP levels already after 5 h of treatment in cells growing on high glucose, while ADP and AMP levels were hardly modified (Fig. 6a). In contrast, in low-glucose medium (YPKG), incubation with MMS did not result in a significant ATP reduction (Fig. 6b). The calculated energy charge (Fig. 6c), a measure of energy stored in adenine nucleotide phosphates [(ATP+1/2ADP)/(ATP+ADP+AMP)], clearly shows a progressive reduction of the cellular energetic pool upon MMS treatment under high-glucose conditions to a value < 0.5. This reduction was mostly
caused by depletion of cellular ATP and only a slight decrease of the ADP pool. When grown in low-glucose/acetate medium (YPKG), the overall energy charge was significantly lower. MMS treatment did not cause a further reduction (Fig. 6c).

The significant decline of the total intracellular pool of adenine nucleotides (AXP) in control samples cultured in both YPD and YPKG media (Fig. 6a and b) during the first hours of incubation is probably a result of adenine nucleotide consumption in DNA and RNA synthesis. At this time point, exponentially growing cells require and rapidly consume large numbers of building blocks. When cells are grown in the low-glucose medium, YPKG, the total intracellular ATP content is slightly lower (accompanied by a higher AMP content), the growth rate is reduced and the reversible decline of the AXP level in the exponentially growing culture is shorter. In treated cells, MMS triggers growth arrest and, therefore, ATP is not consumed in DNA and RNA synthesis, resulting in a constant level of the cellular ATP pool. Prolonged MMS treatment leads to a persistent inhibition of respiratory activity and a diversion of the glycolytic flux, which finally results in an inability of the cells to maintain their energy level and a strong decline of the ATP pool. Our results strongly suggest that the increased resistance of yeast cells to MMS treatment could be related to a better ability of the cells to maintain their energy balance, when grown under calorie restriction conditions.

ROS production and mitochondrial activity in response to MMS treatment

mtDNA undergoes 3–10-fold more damage than chDNA following oxidative stress in numerous cell types from yeast, mouse, rats and humans (reviewed in Van Houten et al., 2006) and it was reported earlier that mtDNA is a preferred target for MMS-mediated DNA damage (Pirsel & Bohr, 1993). mtDNA damage can lead to loss of expression of mitochondrial polypeptides, a subsequent decrease in electron transport and an increase in ROS generation, loss of mitochondrial membrane potential and release of signals for cell death, such as CytC and AIF. By releasing several
proteins that trigger programmed cell death, mitochondria act as the ‘executioners’ in apoptosis (review in Garrido & Kroemer, 2004). We showed earlier that ROS formation upon MMS treatment could be reduced by alterations in glucose metabolism (Kitanovic & Wölfl, 2006). The results presented above indicated that the diauxic shift induced by lower glucose concentrations could prevent inhibition of mitochondrial function upon MMS treatment (preserved CS activity, Fig. 3d) and protect cells from energetic collapse. As mitochondria are major suppliers of ATP and also the major source of ROS, we further investigated mitochondrial features in response to MMS treatment: mitochondrial membrane potential ($\Delta \Psi$), mitochondrial activity and CL.

Fig. 6. (a) Changes in the level of ATP, ADP and AMP upon MMS treatment. Yeast cells were grown in (a) full medium (YPD) and (b) low-glucose/acetate (YPKG) medium until the mid-log phase ($OD_{600nm} = 0.6–1.0$). Cells were treated with 0.03% MMS (MMS) or mock-treated (con). At the indicated time points, aliquots were withdrawn, cells were quickly collected by filtration, quenched in boiling buffered ethanol and the intracellular nucleotide content was analysed by HPLC. The values are presented in $\mu$mol g$^{-1}$ dry weight (DW). AXP = ATP + ADP + AMP. (c) The energy charge was calculated at indicated time points as (ATP + 1/2ADP)/(ATP + ADP + AMP). Graphs present representative experiments of at least three repetitions.
Fluorescence intensities were quantified by flow cytometry. Treatment with 0.03% MMS resulted in a strong induction of ROS and mitochondrial hyperpolarization (Fig. 7a). Under low-glucose conditions, induction of ROS by MMS treatment was significantly lower (Fig. 7a). Treatment with 0.1% MMS over the same time period had a rather opposite effect (Fig. 7b). At this high MMS concentration, neither ROS formation nor significant mitochondrial membrane potential (Ψ) could be detected, most likely reflecting the severe damage that occurred with this highly cytotoxic concentration (Fig. 7b).

It had been shown earlier that a decrease in the metabolic rate could cause specific perturbations of mitochondrial ATP/ADP exchange that results in ATP synthase stagnation and mitochondrial hyperpolarization (Vander Heiden et al., 1999). The inability of F1F0ATPase to utilize the H+ ion gradient facilitates further ROS production and mtDNA damage (Esposito et al., 1999; Vander Heiden et al., 1999). To further analyse mitochondrial function, mitochondria were stained with NAO and Mito Tracker Green FM (MTGreen) (Fig. 8a and b). While NAO specifically stains cardiolipin in the inner mitochondrial membranes, fluorescence staining with Mito Tracker requires mitochondrial activity and is specific for active mitochondria. Dead cells, which could be in particular critical for cardiolipin staining, were excluded from statistical analysis of both NAO and MT Green fluorescence, by costaining with PI.

Long-term treatment of cells with 0.03% MMS (24 h) under high-glucose conditions (Fig. 8a) shows a reduced amount of functional mitochondria. Cultivation under low-glucose conditions prevented the loss of functional mitochondria, which agrees well with the observation that MMS treatment did not cause a significant decline of the ATP level in low glucose media level (Fig. 6b). In both media, addition of MMS led to elevated staining of cardiolipin already after 2 h of treatment, which was even more pronounced in 24-h treated cells (Fig. 8b).

Our results indicate that enhanced mitochondrial capacity may be critical for better tolerance of MMS treatment under low-glucose conditions. Thus, while controlling induction of apoptosis in severely damaged cells, mitochondria are also crucial to maintain the energy supply required for DNA-damage repair and antioxidant defence, enabling cellular survival.

**Oxygen consumption upon MMS treatment**

The mitochondrial function in energy metabolism is directly dependent on oxygen consumption. Therefore, we measured oxygen consumption of MMS-treated cells using oxygen sensor 96-well microtitre plates (PreSens). To test the correlation between glucose availability in the medium and the influence of MMS on mitochondrial respiration, we analysed oxygen consumption of cells treated with 0.01% MMS cultivated at different glucose concentrations (YPD medium with 2%, 1%, 0.5% and 0.25% glucose). The results are summarized in Fig. 9. As expected, biomass production strongly depended on the amount of glucose in the medium, visible as a progressive reduction of biomass yield at lower glucose concentrations (Fig. 9a). MMS treatment caused similar growth inhibition in all cultures. Upon addition of MMS, oxygen consumption of cells was significantly reduced, but resumed at different time points depending on the glucose concentration in the medium (Fig. 9b). At lower glucose levels, the recovery of oxygen consumption was faster, and correlated with glucose depletion from the medium, starting when the glucose concentration declined < 0.1%. Under control conditions, the respiration rate was only slightly influenced by glucose availability, although biomass production correlated directly with the glucose concentrations (Fig. 9).

To test whether MMS directly blocks mitochondrial respiratory activity, we isolated mitochondria from stationary cultures of cells grown in full (YPD) medium. For this, cells were spheroplasted and mitochondria were isolated in ice-cold sorbitol buffer. Mitochondria were resuspended in ice-cold sucrose buffer and mitochondrial activity was stimulated with glutamate/malate and ADP. Oxygen consumption was measured in sorbitol buffer containing MMS (0.01%, 0.02% and 0.04%) or 3 mM \( \text{H}_2\text{O}_2 \) with oxoplates. The results showed that both MMS and \( \text{H}_2\text{O}_2 \) directly block oxygen consumption of isolated mitochondria (Fig. 10). Therefore, we concluded that MMS treatment can directly block mitochondrial respiration, which consequently leads to increased ROS production, induced oxidative stress and inhibition of GAPDH activity.

**The effect of MMS treatment directly depends on the cellular content of respiratory chain compounds and the respiration rate**

Our results suggest that enhanced mitochondrial activity and cellular respiration is beneficial for cellular survival by enabling a fast and better recovery upon MMS treatment. To test this hypothesis, we included treatment with a mitochondrial uncoupling agent, CCCP (carbonyl cyanide m-chlorophenyl hydrazone), and performed experiments in hap4 deletion mutants, in which the synthesis of proteins involved in the TCA cycle, the electron transport chain, ATP generation and mitochondrial biogenesis is impaired (reviewed in Schüller, 2003). To ensure highly energized and functional mitochondria in the cells, the experiments were performed in low-glucose/acetate medium. Addition of CCCP increased oxygen consumption of control cells and promoted faster recovery of mitochondrial activity in...
MMS-treated cells (Fig. 11a). Moreover, the initial block of oxygen consumption was significantly lower in CCCP-containing cultures. In contrast, disruption of the heme-activated protein complex Hap2/3/4/5 through the deletion of HAP4 reduced the speed of oxygen utilization and strongly enhanced the MMS-mediated respiratory block (Fig. 11b). Remarkably, hap4 mutant cells were not able to regain mitochondrial activity and oxygen consumption even upon treatment with lower MMS concentrations (0.01% and 0.02%), conditions under which wild-type cells can overcome respiratory inhibition and continue proliferation. Thus, the results obtained support our hypothesis that high activity of the respiratory chain, which must be even higher in the presence of an uncoupling agent, reduces the development of oxidative stress and subsequent cell death.

Fig. 8. (a) Modulation of mitochondrial bioenergetic conditions of yeast cells in response to MMS treatment. Exponentially growing wild-type cells were treated with 0.03% MMS in full (YPD) or low-glucose (YP-0.5% Glu) media. Samples were withdrawn after 2 h and 24 h of treatment and double-stained with Myto Tracker Green/PI (a) or NAO/PI (b). Histogram plots present the fluorescence intensity of Myto Tracker (a) and NAO staining (b). PI-positive cells are excluded from gates. The mean values of viable cells (non-PI positive) are presented for each measurement. The number of dead cells (excluded from analysis) in each sample is given as % of PI-positive cells.
Discussion

Our study shows that MMS treatment results in a strong and rapid inhibition of oxygen consumption, indicating a clear decrease of respiratory activity, as well as a drastic increase in ROS production. The observed effects on mitochondrial function and ROS production could be based on mtDNA damage, which triggers ROS production (Salmon et al., 2004; Kitanovic & Wölfli, 2006) and further impairs the activity of mitochondrial proteins. This could be shown for CS, whose activity is reduced after longer exposure to MMS. While oxygen consumption is decreased, other markers of mitochondrial activity are less significantly changed or even higher. Measuring oxygen consumption in isolated mitochondria, we could show that inhibition of mitochondrial respiration by MMS is intrinsic to mitochondria, supporting our hypothesis that it could be the primary mode of action for MMS.

A major effect triggered by the alkylating agent MMS on yeast cells was to induce a dramatic metabolic modification indicated by the inability of yeast cultures to shift the metabolism from fermentation to oxidative phosphorylation. As a consequence, the glucose flow is diverted into the glycerol, PPP and glucose stores, glycogen and trehalose, which were consumed later as the sole endogenous carbon source remaining for cell survival. This rapid mobilization of stored glucose from glycogen and trehalose as MMS-treated cells ran out of an exogenous carbon source is consistent with the notion that these cells cannot resume growth on a nonfermentative carbon source and hence must thrive on their endogenous carbon source for energy maintenance (Enjalbert et al., 2000; Jules et al., 2008). As a consequence, the energy charge was strongly reduced with a net depletion of cellular ATP.

The inhibitory effect of MMS treatment on cellular proliferation and energy content was concomitant with persistent glucose consumption and increased glycerol and...
Nitrosation or oxidation in response to H$_2$O$_2$ treatment, S may be subjected to post-translational modification such as but results from the literature indicate that these enzymes 

GAPDH in this specific condition has not been investigated, repressed mitochondria. The mechanism of inhibition of challenge seemed to be the result of high ROS accumulation by mitochondria. Instead, inhibition of GAPDH upon MMS challenge could reflect a high requirement for NADPH in antioxidant defence. MMS has no direct inhibitory effect on these enzymes. Se instead, inhibition of GAPDH upon MMS challenge is strongly inhibited GAPDH and PYK activity independent of the glucose concentration in the medium. In contrast, the activity of G6PDH was strongly enhanced by MMS, which could reflect a high requirement for NADPH in antioxidant defence. MMS has no direct inhibitory effect on these enzymes. Instead, inhibition of GAPDH upon MMS challenge seemed to be the result of high ROS accumulation by repressed mitochondria. The mechanism of inhibition of GAPDH in this specific condition has not been investigated, but results from the literature indicate that these enzymes may be subjected to post-translational modification such as S-nitrosation or oxidation in response to H$_2$O$_2$ treatment,

which results in inhibition of its dehydrogenase activity (Shenton & Grant, 2003; Osório et al., 2004; Almeida et al., 2007). How PYK is repressed remains unclear, because its activity was not affected directly by peroxide in vitro. Interestingly, genetic analysis of yeast PYK showed that it is involved in the cell division cycle, and is also named CDC19. Temperature-sensitive cdc19 mutants arrest growth in G1 at the restrictive temperature of 36 °C (Aon et al., 1995) as a result of carbon metabolism and energy uncoupling. Thus, cell cycle arrest and reduced proliferation by MMS-induced DNA damage is probably enhanced upon PYK inhibition, redirecting carbon flow to glycerol, glycogen and trehalose accumulation.

The concentration of glucose in the medium seems to be a key factor in the response to MMS. Preincubation under low-glucose conditions enables yeast cells to survive much better upon MMS treatment. Inhibition of cellular respiration upon MMS treatment is also directly correlated with the glucose concentration in the medium. At low glucose, the glycolytic rate is reduced, resulting in lower repression of respiration and TCA enzymes, which is reflected in decreased PYK and GAPDH activities and increased CS activity. As a consequence, pyruvate is utilized more effectively in mitochondria, enabling NADH production in the TCA cycle and NAD$^+$ regeneration in the respiratory chain. At high glucose (fermentative growth), pyruvate is mostly converted to ethanol, due to strong repression of mitochondria. This is in accordance with earlier observations that calorie restriction (CR)-mediated metabolic shift results in a more efficient electron transport in the mitochondrial respiratory chain (Weindruch et al., 1986; Sohal & Weindruch, 1996). Faster and more efficient electron transport will enhance oxygen consumption and therefore reduce oxygen concentrations in the mitochondrial microenvironment. Thus, it is quite conceivable that the final outcome of enhanced respiration of calorie-restricted cells when challenged with MMS is reduced leakage of electrons from the respiratory chain and a lower production of ROS. This should improve cellular survival upon MMS treatment, which is the case in our experiments, showing better survival of cells on MMS plates when cells were precultured under CR conditions.

Scavenging of ROS, which increased the survival of MMS-treated cells (Kitanovic & Wölfli, 2006), has a similar effect on survival as caloric restriction. This is in good agreement with the observation that CR decreases the ratio of ROS produced over O$_2$ consumed in isolated mammalian mitochondria (Gredilla et al., 2001). It is also known that CR enhances the expression of mitochondrial uncoupling proteins (Merry, 2002; Bevilacqua et al., 2004), which could be an explanation for the reduced ROS produced/O$_2$ consumed ratio.

These observations suggest that mitochondrial activity and its roles in maintaining cellular energy levels as well as

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**Fig. 11.** (a) Influence of mitochondria respiratory conditions on MMS-induced inhibition of oxygen consumption. Yeast cells were incubated in a full (YPD) or a low-glucose/acetate (YPKG) medium until the mid-log phase (OD$_{600nm}$= 0.6–1.0). Equal amounts of cells were plated in each well of 96-well OxoPlates with a round bottom. Cells were either mock-treated (con) or treated with different MMS concentrations in triplicate. Experiments and calculations were performed as detailed in Fig. 9. (a) Kinetic of oxygen consumption of wild-type cells treated with MMS with or without addition of 5µM CCCP was monitored during 18 h of cultivation in YPKG medium. (b) Oxygen consumption of wild-type (wt) and Δhap4 cells treated with MMS was monitored during 17 h of cultivation in YPD medium.
ROS formation are the critical link between CR and decreased sensitivity to MMS. In fact, analysing mitochondrial parameters, we found that treatment with MMS not only leads to ROS formation but also induces changes in mitochondrial activity. Blocking the last step, in the respiratory chain, oxygen consumption, electrons must be transferred to other targets, leading to ROS formation. In this condition, the membrane potential is increased, despite reduced activity, which may result in mitochondrial reorganization reflected in enhanced cardiolipin staining. This fits very well with a dual role of ROS signalling in response to mitochondrial stress. At low levels, ROS would mediate retrograde signalling inducing synthesis of mitochondrial genes for mitochondrial biogenesis, while in response to severe damage, cell death is induced by high levels of ROS (Piantadosi & Suliman, 2006; Passos et al., 2007). Under CR conditions, the membrane potential and respiratory activity is significantly enhanced to ensure energy and redox homeostasis. Modulation of membrane uncoupling and the need for mitochondrial ATP production ensures constant activity of the respiratory chain and suppresses ROS formation. Thus, when the flux in the respiratory chain is artificially increased by an uncoupling agent, cells should more efficiently tolerate toxic challenges, while suppression of respiratory activity should inhibit the efficient response to the toxic challenge. Indeed, treatment with the uncoupling agent CCCP leads to an improved recovery of respiration, indicating a more efficient flux in the respiratory chain. Reduction of the respiratory capacity, which is the case in the hap4 deletion mutant, in which synthesis of electron transport chain components is repressed (high glucose leads to similar repression) (Lin et al., 2002, 2004; Schüller, 2003), prevents the recovery from MMS treatment. As a result, although the activity of the respiratory chain is decreased, the block of respiration by MMS in this mutant leads to an accumulation of electrons at intermediate levels of the respiratory chain, favouring electron leakage and very high ROS formation (data not shown).

In summary, the above data demonstrate that the cellular metabolic status determines the cellular fate in response to treatment with DNA-damaging agents. High respiratory activity significantly reduces ROS production and thus serves as a protective mechanism against treatment with agents that damage mtDNA. In addition, we could show

![Diagram of yeast metabolism](https://academic.oup.com/femsyr/article-abstract/9/4/535/593530)
that the mechanism by which alkylating agents, such as MMS, induce apoptosis is closely related to inhibition of the lower parts of glycolysis as well as of the mitochondrial respiratory activity, thereby increasing ROS accumulation and induction of oxidative stress (Fig. 12). Promoting the efficient generation of free radicals by respiratory inhibition could enhance the cytotoxic activity of DNA-damaging agents, which could be exploited to increase the efficiency of anticancer therapy. In a recent publication, Raffaghello et al. (2008) showed that starvation-induced stress resistance protected yeast and mammalian cells from oxidative or alkylating agents. Interestingly, they showed that the same conditions did not improve survival of cancer cells. This effect was connected to Ras, Akt and mTor in the nutrient response pathway, which is deregulated in cancer cells. Our results are in direct agreement with this observation. In fact, tumour cells are often defective in antioxidative stress response (reviewed in Verrax et al., 2008) and are highly dependent on glycolysis. Taken together, our results strongly support the idea that preservation of mitochondrial activity is crucial for cellular defence to DNA damage and oxidative stress. Therefore, it should be expected that starvation could selectively increase the resistance of normal cells. Our results also support the possibility of using yeast cells to screen for a drug combination that can sensitize cancer cells to anticancer agents.

**References**


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