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Biomarkers of oxidative stress in the fungal strain *Humicola lutea* under copper exposure

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ABSTRACT

The fungal strain *Humicola lutea* 103 was used as a model organism to examine the relationship between copper toxicity and oxidative stress in low eukaryotes such as filamentous fungi. Spores or submerged cultures were treated with different copper concentrations and the oxidative stress-inducing agent paraquat (PQ). Oxidative stress biomarkers such as reactive oxygen species (ROS), cyanide-resistant respiration, protein carbonyls, reserve carbohydrates, and antioxidant defence were identified in cells treated or not treated with either copper ions or PQ. Copper inhibited the growth and conidiospore formation of *H. lutea* 103 in a concentration-dependent manner. This treatment also resulted in increased superoxide anion radical formation. Copper stress was furthermore accompanied by transient accumulation of trehalose and glycogen, as well as increased protein carbonyl content. Compared to control cultures, copper-treated mycelia demonstrated a marked increase in the activity of protective enzymes (superoxide dismutase, catalase, and glucose-6-phosphate dehydrogenase). These increased antioxidant enzyme activities were blocked by inhibitors of protein synthesis, suggesting that *de novo* enzyme formation was involved. Biomarker response to the heavy metal was similar to treatment with known ROS generators such as PQ. The observed hyper-oxidative status and increased oxidative damage suggest a relationship between acute metal treatment and oxidative stress in fungal cells.

1. Introduction

Reactive oxygen species (ROS) such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) are produced intracellularly in all aerobic organisms and are normally in balance with antioxidant molecules. Oxidative stress occurs when this critical balance is disrupted due to depletion of antioxidants or excess accumulation of ROS [1]. ROS generation can occur via several mechanisms [2]. Regardless of how or where they are generated, increased levels of ROS can damage all types of biological molecules, including DNA, lipids, proteins, and carbohydrates. Such damage influences numerous cellular processes, leading to compromised cell function or to cell death [3].

In response to harmful ROS, aerobically growing organisms (eukaryotic and prokaryotic) have evolved multiple defence mechanisms for protection of their cellular components [4]. The first line of defence against ROS is prevention of their formation. There are also protective proteins that remove ROS, and secondary defences consist of enzymes that remove and repair the products of oxidatively damaged components [5]. Despite the existence of many nonenzymatic anti-oxidant compounds (glutathione, α-tocopherol, ascorbate), the most efficient way to eliminate ROS is through catalysis by antioxidant enzymes such as superoxide dismutase [EC 1.15.1.1.] (SOD), catalase [EC 1.11.1.6.] (CAT), and peroxidases [EC 1.11.1.7.] (POXs) [6].

Environmental stresses such as exposure to heavy metals are known to promote ROS formation in cells, potentially overwhelming antioxidant defences [7]. This is particularly true of redox-active metals like copper (Cu) that catalyze the Fenton reaction and can accelerate generation of highly damaging $OH^{\bullet}$ radicals from $^{\bullet}O_2^-$ and H_2O_2 substrates [8,2]. At the same time, Cu serves an essential role in biological processes because of its catalytic and structural properties. Thus, the maintenance of Cu homeostasis at the cellular level is crucial for aerobic organisms [9]. The phenomenon of Cu activation of ROS production is described as oxidative stress in different models [10,11]. Exposure to this heavy metal provokes a pronounced response of antioxidant systems [11,12].

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In filamentous fungi, defence mechanisms against Cu stress are currently under investigation [13]. Attention has been paid mainly to alterations in the antioxidant defence system in the presence of Cu ions [14,15]. However, the evaluation of events such as ROS generation, oxidative damage to proteins, and biosynthesis of reserve carbohydrates that are typical of both copper and oxidative stress have been seldom performed.

Our previous studies revealed that the fungal strain *Humicola lutea* 103 produces naturally glycosylated Cu/Zn-superoxide dismutase (Cu/Zn-SOD) at high levels [16,17,18]. Such glycosylated SODs could only be isolated in limited cases. *H. lutea* enzymes protected against myeloid Graffi tumours in hamsters and experimental influenza virus infection in mice [16,17]. Our preliminary study showed that addition of Cu ions to productive medium resulted in increased enzyme activity. Thus, the Cu stress response in *H. lutea* cultures might be of industrial interest.

The aim of the present investigation was to compare the fungal cell response against copper toxicity and oxidative stress in low eukaryotes such as filamentous fungi. Specifically, we investigated the effect of increased concentrations of Cu ions on biomarkers of cyanide-resistant respiration (CNRR), ROS, carbonyl groups, and reserve carbohydrates, as well as on the growth of *H. lutea* cultures. We present evidence indicating that responses to Cu treatment resemble those caused by oxidative stress-inducing agents. This report also suggests a relevant role for antioxidant enzymes in Cu resistance.

2. Materials and methods

2.1. Materials

Paraquat (PQ), nitro blue tetrazolium (NBT), cytochrome *c*, horseradish peroxidase type VI-A, NADPH, α-amyloglucosidase from *Aspergillus niger*, trehalase, 2,4-dinitrophenylhydrazine (DNPH), hydroxyurea (HU), actinomycin D (AD), 8-azaguanine (8-AG), and cycloheximide (CHI) were obtained from Sigma–Aldrich (Deisenhofen, Germany). All other chemicals used in this study were of the highest analytical grade.

2.2. Fungal strain and culture conditions

The fungal strain *H. lutea* 103 from the Mycological Collection at the Institute of Microbiology, Sofia, was used throughout and maintained at 4 °C on beer agar, pH 6.3. To monitor the Cu-resistance of the fungal strain, conidiospores were cultivated in Petri dishes (d = 10 mm) with beer agar supplemented with various concentrations of $CuSO_4$ for 10 days at a temperature of 30 °C.

For submerged cultivation, both seed and productive media were used [19]. For the inoculum, 80 ml of seed medium was inoculated with 5 ml of spore suspension at a concentration of 2×10^8 spores ml^{-1} in 500 ml Erlenmeyer flasks. Cultivation was performed on a shaker (220 rpm) at 30 °C for 24 h. Then, 6 ml of seed culture were transferred to 500 ml Erlenmeyer flasks containing 74 ml of production medium. The cultures were grown at 30 °C for 96 h. For investigation of the effects of different Cu concentrations, cultures were incubated with 3 mM PQ or various concentrations of $CuSO_4$ in order to achieve 40, 70, 100, 150, and 300 μg/ml Cu ions. Results were evaluated from repeated experiments using three or five parallel runs.

For some experiments, non-growing mycelium was used. In these cases cells were cultivated for 24 h (mid-logarithmic growth phase) in the seed medium as described above. Then, 1 g of wet mycelium was added to 40 ml of medium III (KH_2PO_4: 5 g/l and $MgSO_4 \cdot 7H_2O$: 2.5 g/l, pH 7.8) with or without stress-inducing agents (3 mM PQ or 40, 70, 100, 150, and 300 μg/ml Cu ions) in 500 ml Erlenmeyer flasks, followed by incubation at 30 °C on a shaker (220 rev/min) for 30, 60, and 120 min.

For experiments concerning inhibition of protein synthesis, non-growing cells were cultivated in the presence of 180 μg/ml actinomycin D, 500 μg/ml hydroxyurea, 150 μg/ml 8-azaguanine, or 180 μg/ml cycloheximide for 1 h pre-cultivation. This time was necessary for penetration of the inhibitors into the fungal cells. After pre-cultivation, the mycelium was treated with Cu ions or PQ for 3 h.

2.3. Cell-free extract preparation and isolation of mitochondria

All steps during the isolation of mitochondria were performed at 0–4 °C. Mycelial disruption and mitochondrial isolation were performed according to Lambowitz [20] with some modifications. Cells from 24 h cultures were harvested using a filter paper-covered funnel (Whatman No. 4 filter, Clifton, USA) with a sieve connected to a vacuum pump, and washed repeatedly on the filter with distilled water and then once with isolation buffer (0.25 M sucrose, 5 mM EDTA, 0.15% bovine serum albumin, pH 7.5). For every 10 g of wet weight hyphae, 5 g of quartz sand and 20 ml of the same buffer containing 0.3 mM phenylmethylsulfonyl fluoride (PMSF) were added to the mortar and ground together for 1–2 min at 4 °C. After addition of another 10 ml of the same buffer, the slurry was centrifuged 3 times at 1500 × g for 15 min to remove the quartz sand and undisrupted cells. The resulting supernatant was further centrifuged at 15,000 × g for 30 min and the liquid fraction was used as the cell-free extract (CFE). The 15,000 × g precipitate was resuspended in the same volume of isolation buffer and centrifuged for 40 min at 30,590 × g. Then, the supernatant was discarded and the crude mitochondrial pellet (CMP) was carefully washed with SEM buffer (0.25 M sucrose; 5 mM EDTA; 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS)/KOH, pH 7.5). This procedure was repeated three times, and the collected mitochondria were resuspended in SEM buffer. For measurement of ROS generation, the mitochondria were used immediately following their isolation.

2.4. Enzyme activity determination

SOD activity was measured in CFE by NBT reduction [21]. One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units per mg protein (U/mg protein). Cyanide (5 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/Zn-SOD and cyanide-resistant Mn SOD. Cu/Zn-SOD activity was obtained as total activity minus the activity in the presence of 5 mM cyanide. Catalase was assayed by the method of Beers and Sizer [22], in which the decomposition of H_2O_2 was analysed spectrophotometrically at 240 nm. One unit of catalase activity was defined as the amount of enzyme that decomposes 1 μmol H_2O_2/min at an initial H_2O_2 concentration of 30 mM at pH 7.0 and 25 °C. Glucose 6-phosphate dehydrogenase (G6PD) activity was measured by glucose 6-phosphate-dependent reduction of $NADP^+$ [23]. One unit was equivalent to 1 μmol of substrate reduced per min. The specific activity is given as U/mg protein.

2.5. Measurement of total and cyanide-resistant respiration

Oxygen uptake by fungal cell suspensions treated or not treated with Cu or PQ was measured at 30 °C in the absence and presence of 10 mM cyanide by using an oxygen meter, type 5221-ELWRO (Poland), and expressed as milligrams O_2 per gram dry weight per minute. The measurement was carried out as previously described [24].

2.6. Determination of ROS

For measurement of $^{\bullet}O_2^-$ production rate, the method of superoxide dismutase-inhibitable reduction of cytochrome *c* was used [25] with some modifications. Briefly, cell suspensions or crude mitochondrial pellets taken from 24 h cultures grown in the presence of 3 mM PQ or various Cu^{2+} concentrations, were incubated for 60 min at 30 °C on a water bath rotary shaker at 150 rpm. The reaction mixtures contained 50 μM cytochrome *c*, 2% non-autoclaved glucose, and 20 mM NADPH in the presence and absence of 50 μg ml^{-1} of superoxide dismutase from bovine erythrocytes in 0.05 M potassium phosphate buffer at pH 7.8. The reaction was stopped by cooling in an ice-cold water bath. The cells were removed by centrifugation before reading the absorbance at 550 nm to determine the extent of cytochrome *c* reduction. A molar extinction coefficient of 2.11×10^4 was used to calculate the concentration of reduced cytochrome *c*.

For measurement of hydrogen peroxide production, the method of Pick and Mizel [26] was used. Briefly, fungal cells or CMP were suspended in 0.05 M potassium phosphate buffer at pH 7.8 containing 50 μg ml^{-1} horseradish peroxidase type VI-A. After incubation at 30 °C for 45 min, the reaction was stopped by addition of 1N NaOH, and the absorbance was read at 620 nm. For calculations, a standard curve with H_2O_2 concentrations (from 5 to 50 μmol) was used.

2.7. Measurement of protein carbonyl content

Protein oxidative damage was measured spectrophotometrically as protein carbonyl content using the DNPH binding assay [27], slightly modified by Adachi and Ishii [28]. Following Cu treatment, the cell-free extracts were incubated with DNPH for 1 h at 37 °C; proteins were precipitated in 10% cold TCA and washed with ethanol:ethylacetate (1:1), to remove excess DNPH and finally dissolved in 6 M guanidine chloride, pH 2. The optimal density was measured at 380 nm, and the carbonyl content was calculated using a molar extinction coefficient of 21 mM^{-1} cm^{-1}, resulting in final measurement of nanomoles of DNPH incorporated (protein carbonyls) per mg of protein.

2.8. Determination of reserve carbohydrates

In order to determine glycogen and trehalose content, a procedure previously described by Becker [29] and Vandecamen et al. [30] and then modified by Parrou et al. [31] was used. Soluble reducing sugars were determined by the Somogy-Nelson method [32].