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Impact of oxidative stress on *Acanthamoeba castellanii* mitochondrial bioenergetics depends on cell growth stage

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Abstract Addition of a moderate (1.4 mM) concentration of H_2O_2 to protozoon *Acanthamoeba castellanii* cell cultures at different growth phases caused a different response to oxidative stress. H_2O_2 treatment of exponentially growing cells significantly delayed their growth; however, in mitochondria isolated from these cells, no damage to their bioenergetic function was observed. In contrast, addition of H_2O_2 to *A. castellanii* cells approaching the stationary phase did not influence their growth and viability while seriously affecting mitochondrial bioenergetic function. Although mitochondrial integrity was maintained, oxidative damage was revealed in the reduction of cytochrome pathway activity, uncoupling protein activity, and the efficiency of oxidative phosphorylation as well as the membrane potential and the endogenous ubiquinone reduction level of the resting state. An increase in the alternative oxidase protein level and activity as well as an increase in the membranous ubiquinone content were observed in mitochondria isolated from late H_2O_2-treated cells. For the first time, the regulation of ubiquinone content in the inner mitochondrial membrane is shown to play a role in the response to oxidative stress. A physiological role for the higher activity of the alternative oxidase in response to oxidative stress in unicellular organisms, such as amoeba *A. castellanii*, is discussed.

Keywords *Acanthamoeba castellanii* · Alternative oxidase · Hydrogen peroxide · Oxidative stress · Mitochondria · Ubiquinone · Uncoupling protein

Abbreviations

AcAOX	Alternative oxidase of *Acanthamoeba castellanii*
AcUCP	Uncoupling protein of *Acanthamoeba castellanii*
AOX	Alternative oxidase
BHAM	Benzohydroxamate
BSA	Bovine serum albumin
COX	Cytochrome *c* oxidase
$\Delta\mu H^+$	Proton electrochemical gradient
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
ROS	Reactive oxygen species
state 3	Phosphorylating respiration (in the presence of ADP)
state 4	Resting respiration (in the absence of added ADP)
RCR	Respiratory control ratio (state 3/state 4)
TMPD	N,N,N′N′-tetramethyl-*p*-phenylenediamine
UCP	Uncoupling protein
Q	Ubiquinone (coenzyme Q)
QH_2	Reduced Q (ubiquinol)
Qtot	Total pool of endogenous Q in the inner mitochondrial membrane
QH_2/Qtot	Reduction level of Q

Introduction

Mitochondria are the key cellular source of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). The oxidative stress

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resulting from an increase in the generation of reactive oxygen species (ROS) leads to damage of mitochondrial DNA, proteins, membranes, acceleration of aging, and in many cases, to cell death (Papa and Skulachev 1997). Hydrogen peroxide is damaging to mitochondria and other cell components because it can oxidize biomolecules directly or through the formation of hydroxyl radical. The consequences of elevated mitochondrial H_2O_2 concentrations include protein and lipid peroxidation, cytochrome *c* release, inactivation of respiratory chain carriers and other mitochondrial enzymes, and uncoupling or decrease in respiration (Vladimirov et al. 1980; Zhang et al. 1990; Radi et al. 1993; Sweetlove et al. 2002).

Acanthamoeba castellanii is a small, non-photosynthesizing free-living amoeba found in soil and water environments. *A. castellanii* is also an opportunistic pathogen of clinical interest that is responsible for several distinct human diseases. In molecular phylogenesis, *A. castellanii* appears on a branch basal to the divergence points of plants, animals, and fungi (Wainright et al. 1993). Under axenic non-pathogenic conditions, *A. castellanii* has been used as a model organism to study mitochondrial energy-dissipating systems, such as a cyanide-resistant alternative oxidase (AcAOX) (Jarmuszkiewicz et al. 2005a; Woyda-Ploszczyca et al. 2009), an ATP-sensitive potassium channel (Kicinska et al. 2007), and an uncoupling protein (AcUCP) (Jarmuszkiewicz et al. 2010). The mitochondria of *A. castellanii* contain a plant-type respiratory chain with additional (in addition to the four classical) electron carriers in the form of external and internal NADH dehydrogenases and an alternative cyanide-resistant quinol oxidase (AOX) that consumes mitochondrial reducing power without energy conservation in the proton electrochemical gradient (Jarmuszkiewicz et al. 1997). We have shown that in *A. castellanii,* as in plant mitochondria (Popov 2003; Vercesi et al. 2006), the two mitochondrial energy-dissipating systems, AcAOX and AcUCP, may play a role in the energetic status of the cell by decreasing the yield of ATP synthesis and in attenuating ROS production (Czarna and Jarmuszkiewicz 2005; Czarna et al. 2007). Moreover, the contribution of both energy-dissipating systems in the prevention of mitochondrial ROS generation in vivo could ensure their constant level throughout the growth cycle of the *A. castellanii* batch culture (Czarna et al. 2007). It has been shown that both AcUCP and AcAOX could be cold response proteins in unicellular organisms because cold treatment of amoeba cultures increases their activity and protein level (Jarmuszkiewicz et al. 2001, 2004).

We have observed previously that short (5 min) incubation of isolated *A. castellanii* mitochondria with H_2O_2 (up to 25 mM) did not significantly damage their basic energetics (Jarmuszkiewicz et al. 2008). In the present study, cell cultures were treated with H_2O_2. to overcome the remarkable resistance of *A. castellanii* mitochondria to oxidative damage. The aim of the present work was to establish a response of *A. castellanii* mitochondria to oxidative stress caused by H_2O_2 added to cell batch cultures at different phases of growth, i.e., during intensive cells division at inoculation (at the exponential phase of growth) and during slowing cell division as the stationary phase of growth is approached. We measured changes in mitochondrial bioenergetics, including mitochondrial respiration through the energy-conserving (the cytochrome pathway) and energy-dissipating (the AcUCP-sustained respiration and AcAOX-mediated respiration) systems, the efficiency of oxidative phosphorylation, and outer mitochondrial membrane integrity.

Materials and methods

Cell culture and mitochondrial isolation

The soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured as described previously (Czarna et al. 2007). After 72 h, cells were inoculated (time 0) to a final density of approximately $3.5 \pm 0.2 \times 10^5$ cell/ml (Fig. 1). After about 40 h of exponential growth with a generation time (a cell doubling time) of 7–8 h, amoeba cultures reach the intermediary phase and then the stationary phase, and the latter stage precedes transformation into cysts within a few hours. Cell cultures were treated with 1.4 mM H_2O_2 added once at 48 h following inoculation (late H_2O_2-treated cells)

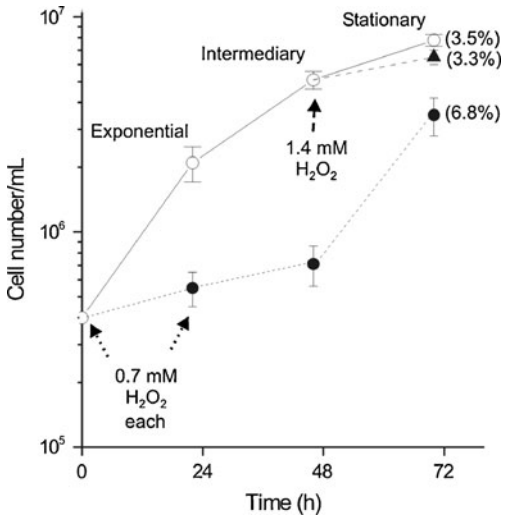


Fig. 1 Growth of *A. castellanii* cells in agitated batch cultures at 28 °C. Cell numbers were determined at the specified intervals. Mean values (± SE) from three separate experiments are shown. Additions of H_2O_2 are indicated. The percentage of dead cells in the *A. castellanii* batch culture is shown in *brackets*

or in two portions of 0.7 mM H_2O_2 added first at inoculation and then at 24 h following inoculation (early H_2O_2-treated cells) (Fig. 1). Trophozoites of *A. castellanii* were harvested from cultures at 72 h following inoculation at the following densities: control untreated cells, $7.8 \pm 0.7 \times 10^6$ cell/ml; late H_2O_2-treated cells, $6.5 \pm 0.8 \times 10^6$ cells/ml; and early H_2O_2-treated cells, $3.5 \pm 0.4 \times 10^6$ cell/ml.

Mitochondria were isolated by differential centrifugation and purified on a self-generating Percoll gradient (30%) as described previously (Jarmuszkiewicz et al. 1997). The depletion of endogenous free fatty acids in the mitochondrial preparations was ensured by the presence of 0.4% fatty acid-free bovine serum albumin (BSA) in the isolation media except for the last mitochondrial washing (Jarmuszkiewicz et al. 1999). The mitochondrial protein concentration was determined using the biuret method using BSA as a standard.

Cell viability

A. castellanii cells were incubated at a 1:1 ratio with 0.05% (*w*/*v*) Evans blue solution. After addition of the dye, the cells were incubated 15 min at 25 °C and then placed on a counting chamber (hemocytometer). Observations were made using a normal, transmitted light microscope (Carl Zeiss, Germany), and the counts of blue-stained versus non-stained bright-yellowish cells were scored separately.

Oxygen uptake

Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge, UK) oxygen electrode or a Hansatech oxygen electrode with 1 mM NADH, 7 mM succinate or 7 mM malate as respiratory substrates in 3 ml or 1.5 ml, respectively, of the standard reaction medium (25 °C) containing 120 mM KCl, 20 mM Tris–HCl, pH 7.4, 3 mM KH_2PO_4, 2 mM $MgCl_2$, plus or minus 0.1% BSA (fatty acid free), and 3 or 1.5 mg of mitochondrial protein to maintain a concentration of 1 $mg \times ml^{-1}$. Values for O_2 uptake are presented in nmol $O \times min^{-1} \times mg^{-1}$ protein.

State 3 measurements were performed in the presence of 2 mM or 200 μM (pulse) ADP. For ADP/O ratio calculations, the total amount of oxygen consumed during pulse state 3 respiration was used. To inhibit the AcAOX and cytochrome pathway activities, 2 mM benzoxydroxamate (BHAM) and 1.5 mM cyanide, respectively, were used. The AcUCP activity was inhibited with 2 mM GTP. To activate AcAOX, 1 mM GMP was supplied. Different concentrations (up to 20 μM) of linoleic acid, which is the most efficient activator of AcUCP (Swida et al. 2007), were used to activate AcUCP and thereby the UCP activity-sustained respiration. To exclude the activity of the ATP/ADP antiporter, 1 μM carboxyatractyloside was used in state 4 measurements.

The cytochrome *c* oxidase (COX) maximal activity was assessed with 0.25 mg of mitochondrial protein without exogenously added respiratory substrate and in the presence of antimycin A (4 μg/mg of mitochondrial protein), 8 mM ascorbate, 0.06% cytochrome *c*, and up to 2.5 mM N,N,N′ N′-tetramethyl-*p*-phenylenediamine (TMPD). The rate of oxygen consumption following the addition of TMPD reflected the maximal O_2 consumption by COX (complex IV). Outer mitochondrial membrane integrity was assayed as the latency of COX activity during the same measurements, i.e., the acceleration of respiration by addition of cytochrome *c* prior to addition of TMPD.

Membrane potential

The membrane potential (ΔΨ) of the mitochondria was measured simultaneously with the oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo et al. (1979). To calculate the ΔΨ value the matrix volume of amoeba mitochondria was assumed for 2.0 μl mg^{-1} protein. The calculation assumes that TPP^+ distribution between the mitochondria and the medium followed the Nernst equation. ΔΨ values are presented in mV.

Determination of the amount of ubiquinone

The endogenous pool of ubiquinone (Q) in the inner mitochondrial membrane was determined by an extraction technique (from 1 to 2 mg of mitochondrial protein), followed by HPLC detection (Czarna et al. 2007). The endogenous ubiquinone in *A. castellanii* mitochondria is Q-9 (Jarmuszkiewicz et al. 1998). For peak calibration, commercial Q-9 (Sigma) was used. Q reduction levels are expressed as a percentage of total Q (QH_2/Qtot).

SDS-PAGE and immunoblotting

Up to 200 μg of mitochondrial protein (stored in −80 °C) was solubilized in the sample buffer containing 2% (*w*/*v*) SDS, 80 mM Tris–HCl (pH 6.8), 10% glycerol, 0.04% (m/v) bromophenol blue and 2–5% mercaptoethanol (for AcAOX detection, 40 mM DTT was added) and boiled for 5 min. Electrophoresis (SDS-PAGE) was carried out using a 5% polyacrylamide stacking gel and a 12–16% polyacrylamide resolving gel with 4.5 M urea followed by Western blotting. Prestained low-molecular-weight markers were used. Antibodies against AOX of *S. guttatum* (generously supplied by Dr. T.E. Elthon) were used at dilutions of 1:1000. Antibodies raised against UCP3 of human skeletal muscle (Alpha Diagnostic, UCP34-A) were used at dilutions of 1:500. Antibodies against subunit III of yeast cytochrome *c* oxidase (COX III, Molecular Probes, A6408) were used at dilutions of 1:1000. Protein bands were visualized using the Amersham

ECL system and quantified digitally. Detection of COX III, which was present as a constant amount throughout culture growth, is considered to be the loading control for detection of the other proteins.

Protein carbonyl assay

Carbonylated proteins were analyzed using the oxyblot kit according to the manufacturer's instructions (Oxyblot, Millipore). Briefly, mitochondrial samples (10 μg per lane) were treated with 10 mM 2,4-dinitrophenolohydrazine in 2 M HCl, incubated at room temperature and neutralized. The derivatized proteins were separated using SDS-PAGE, transferred to a nitrocellulose membrane and treated as previously described for Western blotting (see above). The primary antibody used was against 2,4-dinitrophenol, and detection was performed using the ECl reagent.

Chemicals

Hydrogen peroxide (Sigma, INFARM) concentrations were determined using a molar absorption coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.

Results

Growth condition of *A. castellanii* batch culture

The principal objective of the present study was to describe the response of *A. castellanii* mitochondria to oxidative stress caused by H_2O_2 added to batch cultures at different phases of growth, i.e., during intensive cell division and during slowing cell division as the stationary phase is approached preceding encystment. A total concentration of 1.4 mM H_2O_2 was chosen because higher concentrations (above 2 mM) led to the complete inhibition of *A. castellanii* cell growth and severe cell damage, from which isolation of mitochondria was impossible. Therefore, separate two additions of 0.7 mM H_2O_2 at inoculation and at the exponential phase (early H_2O_2-treated cells) or a single addition of 1.4 mM H_2O_2 at the intermediary phase (late H_2O_2-treated cells) were performed (Fig. 1). In the case of early H_2O_2-treated cells, a significant delay in the growth of the *A. castellanii* batch culture was observed until 48 h following inoculation. After this period, cells restarted intensive division and reached the density of a late exponential phase of growth ($\sim 3.5 \pm 0.4 \times 10^6$ cell/ml). The percentage content of dead cells when trophozoites were harvested for isolation of the mitochondria (at 72 h following inoculation) was approximately double that of the control cells. Addition of H_2O_2 at the intermediary phase (late H_2O_2-treated cells) did not considerably influence the growth of the batch culture or the amount of dead cells.

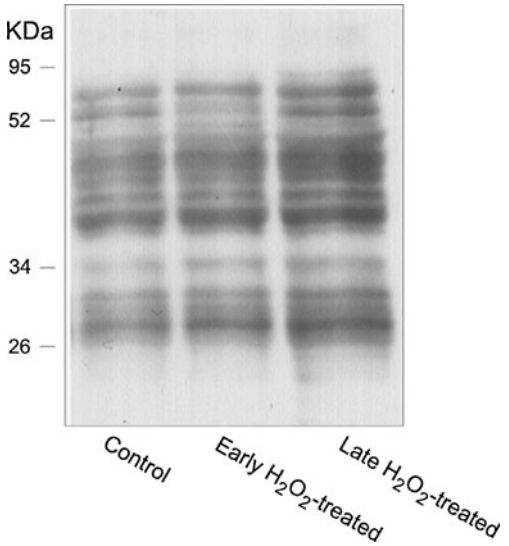
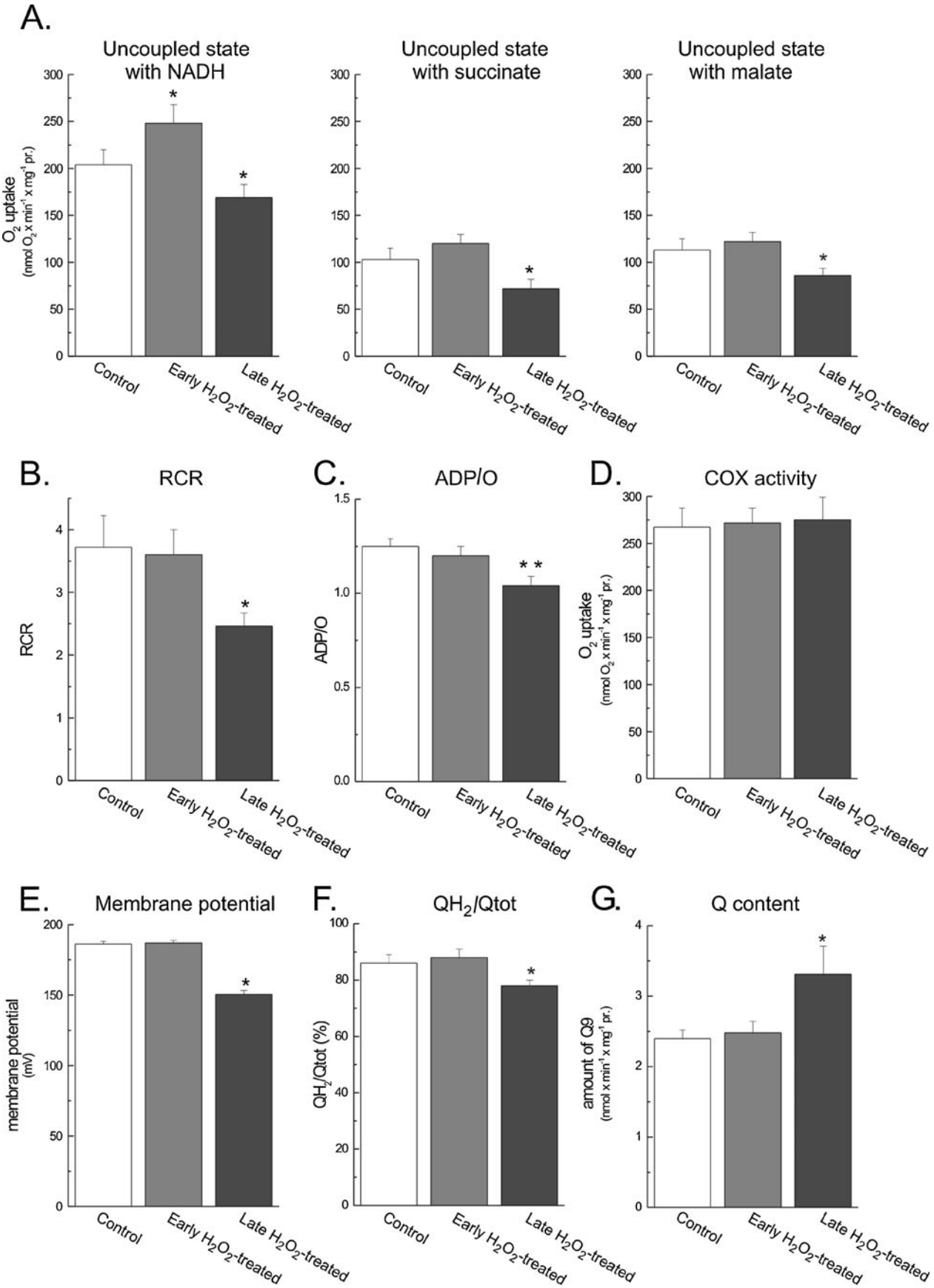


Fig. 2 Levels of oxidized proteins in mitochondria isolated from *A. castellanii* cultures grown in the absence (control cells) or presence of H_2O_2 (early and late H_2O_2-treated cells). Examples of three oxyblots using mitochondria from different preparations are shown. The mitochondrial protein equivalent of 40 μg was loaded in all lines. The gel was stained with oxyblot kit

To evaluate whether H_2O_2 treatment of the *A. castellanii* culture influences the level of oxidized proteins in the mitochondria, we examined the isolated mitochondria for protein byproducts modified by oxidative damage (Fig. 2). Analysis of the mitochondria lysates revealed significantly elevated levels of protein carbonyls, which is a general marker of oxidative damage, in mitochondria isolated from late H_2O_2-treated cells relative to the control cells. In mitochondria isolated from early H_2O_2-treated cells, no increase or a slight increase in oxidized proteins was observed, indicating that the 24 h regeneration period of intensive cell division following 48 h of H_2O_2-induced stress led to elimination or reduction of oxidative damage caused by H_2O_2.

Fig. 3 Cytochrome pathway capacity in mitochondria isolated from *A. castellanii* cultures grown in the presence of H_2O_2 during intensive cell devision (early H_2O_2-treated cells) and during slowing cell division (late H_2O_2-treated cells). **a** For uncoupled respiration measurements with 1 μM FCCP, 0.1% BSA, 2 mM BHAM, 0.5 μM oligomycin, 1 μM carboxyatractyloside, and either 1 mM NADH (plus 4 μM rotenone), 7 mM succinate (4 μM rotenone) or 7 mM malate were supplied. **b**, **c**. Coupling parameters were obtained when state 3 measurements were performed with 200 μM ADP pulses in the presence of 0.1% BSA, 2 mM BHAM, and 1 mM NADH as a respiratory substrate. RCR refers to respiratory control ratios. **d** COX capacity measurements. **e** and **f** Membrane potential and Q reduction level of resting (non-phosphorylating) mitochondria oxidizing NADH. **g** Changes in the amount of total ubiquinone (Q9). The data are the mean (± SE) of five different mitochondrial preparations (n=9–10). *, ** Where indicated, p values are significantly different from control values ($p<0.05$ or $p<0.01$, respectively; Student's unpaired t test)


A.
Uncoupled state with NADH
Uncoupled state with succinate
Uncoupled state with malate
O2 uptake (nmol O2 x min-1 x mg-1 pr.)
B.
RCR
C.
ADP/O
D.
COX activity
E.
Membrane potential
membrane potential (mV)
F.
QH2/Qtot
QH2/Qtot (%)
G.
Q content
amount of Q9 (nmol x min-1 x mg-1 pr.)
Control
Early H2O2-treated
Late H2O2-treated

Cytochrome pathway components and capacity

The capacity for cytochrome pathway-dependent respiration was measured in the presence of 1 μM FCCP (an uncoupler). To exclude the activity of AcAOX and AcUCP, measurements were carried out in the presence of BHAM, which is an inhibitor of AOX, and BSA, which chelates free fatty acids. The results obtained with external NADH (plus rotenone), succinate (plus rotenone) or malate as respiratory substrates are shown in Fig. 3a. Coupling parameters, i.e., the respiratory control ratio (RCR) and ADP/O as well as the membrane potential of resting (non-phosphorylating) mitochondria were measured with external NADH (Fig. 3b–d). External NADH was chosen instead of complex I or complex II substrates to avoid limitations on the cytochrome pathway capacity by these two complexes (Jarmuszkiewicz et al. 2002; Czarna et al. 2007). Additionally, the activity of cytochrome *c* oxidase (complex IV) and the amount of total membranous ubiquinone (Q) were measured (Fig. 3e, f).

In the *A. castellanii* mitochondria isolated from cultures grown in the presence of two portions of 0.7 mM H_2O_2 added during intensive cell division (early H_2O_2-treated cells), none of the tested cytochrome pathway properties changed significantly except for a slight increase in the external NADH-supported uncoupled respiration (Fig. 3). These results indicate that the 48 h period of H_2O_2-induced stress, which was followed by 24 h regeneration period during which the cells underwent intensive division, led to the delay of cell growth reaching the late exponential phase instead of the stationary phase (Fig. 1). Mitochondria isolated from early H_2O_2-treated cells displayed the bioenergetic properties of mitochondria from the late exponential phase (Czarna et al. 2007), and thus, no damage to their function was observed.

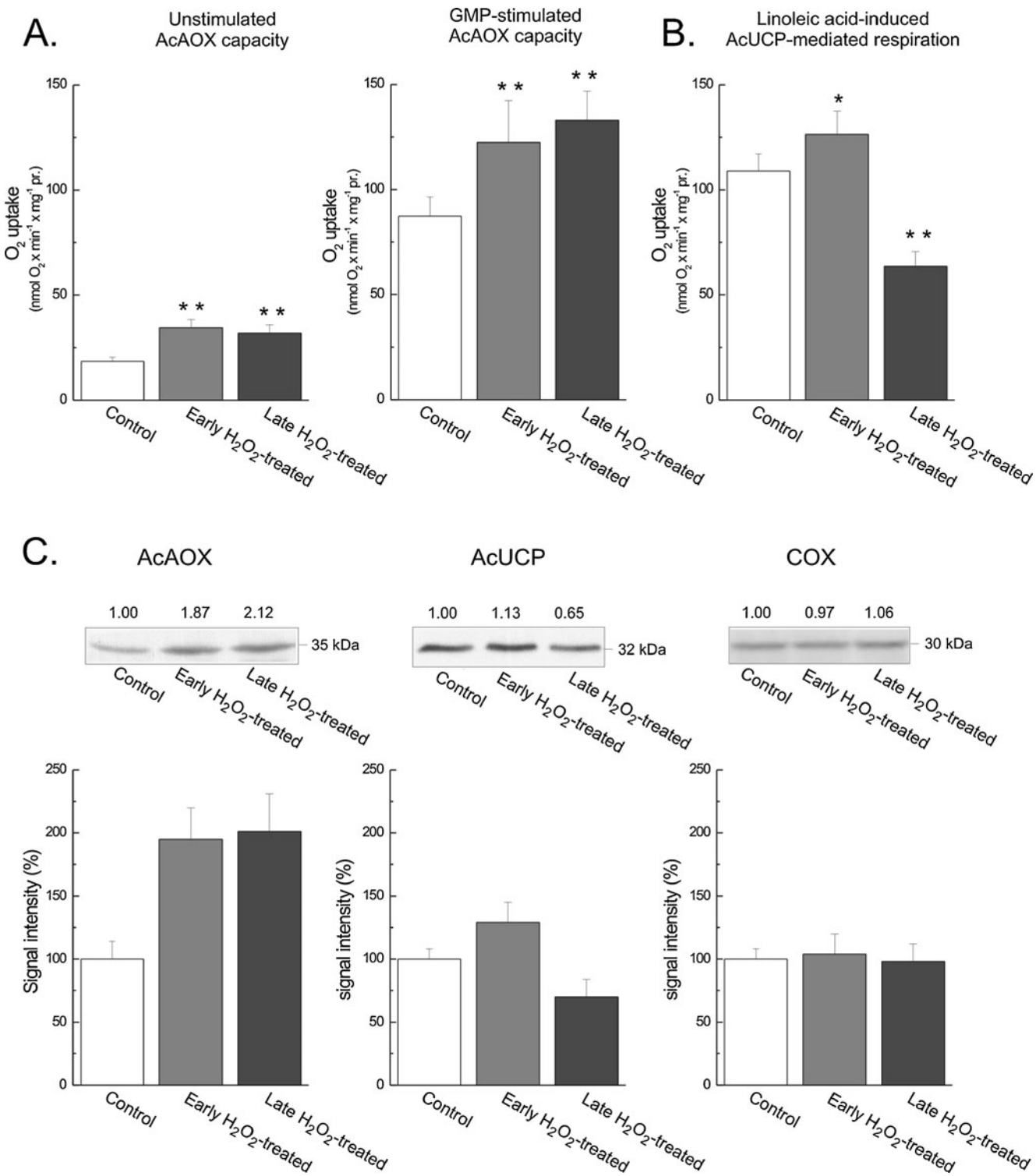
In contrast to the early H_2O_2-treated cells, growth of *A. castellanii* cells in the presence of 1.4 mM H_2O_2 added during slowing cell division (at the intermediary phase, late H_2O_2-treated cells) was associated with marked changes in the cytochrome pathway (complex III and complex IV) activity, i.e., the QH_2-oxygen oxido-reductase activity (Fig. 3). In mitochondria isolated from late H_2O_2-treated cells, the rates of uncoupled (FCCP-stimulated) respiration using either NADH, succinate or malate as the respiratory substrate decreased significantly, and this effect was independent of the supplied respiratory substrate (Fig. 3a). Coupling parameters, i.e., respiratory control ratios (RCR) and ADP/O values (Fig. 3b, c), as well as the membrane potential (Fig. 3e) and endogenous Q reduction level (Fig. 3f) of the resting (non-phosphorylating) state were also lowered. A considerable increase (around 38%) in the amount of membranous Q was observed (Fig. 3g). However, no differences were observed in non-phosphorylating state 4 respiration (data not shown), indicating that external NADH dehydrogenase activity was not damaged under H_2O_2-induced stress conditions. In addition, no change in the amount of complex IV protein (Fig. 3) or in its activity (Fig. 2) was observed, indicating that the terminal oxidase of the *A. castellanii* respiratory chain is not rate limiting for the cytochrome pathway-sustained respiration in mitochondria from both early and late H_2O_2-treated cells. The respiratory rate with exogenous cytochrome *c* as the respiratory substrate was not significantly different in any of the tested mitochondria, indicating that the stress-induced inhibition observed in mitochondria from late H_2O_2-treated cells occurred before the cytochrome *c* oxidase (complex IV) step of the respiratory chain. Thus, the lower respiratory capacity of the cytochrome pathway observed in mitochondria of late H_2O_2-treated cells likely results from reduced activity of complex III.

No significant difference in the outer mitochondrial membrane integrity of *A. castellanii* mitochondria was found between mitochondria isolated from control and stressed cells. Specifically, the outer mitochondrial membrane integrity averaged 98±2%, 98±4%, and 94±5% for mitochondria isolated from control, early and late H_2O_2-treated cells, respectively (for five different mitochondria preparations).

Energy-dissipating systems: AcAOX and AcUCP

Changes in the AcAOX-mediated respiration with NADH as the respiratory substrate were also examined via measurement of the cyanide-resistant respiration in the absence (unstimulated capacity) or in the presence of the most efficient activator of the enzyme (1 mM GMP) (stimulated capacity). A significant (at least 50%) increase in the stimulated and unstimulated AcAOX capacity (Fig. 4a) and in the AcAOX protein level (Fig. 4c) was

Fig. 4 Energy-dissipating systems in mitochondria isolated from *A. castellanii* cultures grown in the absence (control cells) or presence of H_2O_2 (early and late H_2O_2-treated cells). **a** AcAOX activity was measured as cyanide-resistant respiration in the presence of 1 mM NADH as the respiratory substrate, 2 mM cyanide, 0.1% BSA, 1 μM carboxyatractyloside, 0.5 μM oligomycin, plus or minus 1 mM GMP. **b** AcUCP activity (linoleic acid-induced respiration) was measured in the presence of 1 mM NADH, 2 mM BHAM, 1 μM carboxyatractyloside, 0.5 μM oligomycin, and increasing LA concentrations (up to 20 μM). The reported linoleic acid-induced respiration is the difference between respiration observed in the presence of the most effective concentration of linoleic acid and the respiration measured before its addition. **a** and **b** The data shown represent five different mitochondrial preparations for the given conditions. The mean values ± SE are shown (n=6). *, ** Where indicated, p values are significantly different from control values ($p<0.05$ or $p<0.01$, respectively; Student's unpaired t test). **c** Immunodetection of AcAOX and AcUCP proteins. The mitochondrial protein equivalent of 100 μg was loaded in all lines. Examples of four immunoblots, using mitochondria from different preparations, are shown. The highest intensity band was set at "1" and other values are calculated relative to that value. Below, the results of the densitometric analysis are expressed in arbitrary units



observed in mitochondria isolated from early and late H_2O_2-treated cells when compared to control mitochondria. The enhanced rate of stimulated and unstimulated cyanide-resistant respiration was also observed with succinate and malate as respiratory substrates (data not shown).

Figure 4a shows the effect of H_2O_2 treatment of the *A. castellanii* culture on the linoleic acid-induced mitochondrial respiration that represents the AcUCP capacity. The AcUCP-sustained respiration activated by linoleic acid (maximal stimulation at 20 μM) was measured in state 4

in the presence of carboxyatractyloside to exclude ATP/ADP antiporter and in the presence of BHAM to inhibit AcAOX. For mitochondria isolated from early H_2O_2-treated cells, i.e., when H_2O_2 was added during intensive cell division, a less pronounced, approximately 20% increase was observed in the AcUCP capacity (Fig. 4b) and protein level (Fig. 4c) compared to the AcAOX results. In contrast, for mitochondria isolated from late H_2O_2-treated cells, a significant (at least 30%) decline in the linoleic acid-induced AcUCP-sustained respiration (Fig. 4b) and AcUCP protein level (Fig. 4c) was observed when compared to control mitochondria isolated from untreated cells.

In the *A. castellanii* mitochondria isolated from cultures grown in the presence of H_2O_2 added during intensive cell division (early H_2O_2-treated cells), concomitant increases in the activity and protein levels of both energy-dissipating systems (AcAOX and AcUCP) are consistent with our interpretation that these cells are counterparts of control cells from late exponential phase and that these cells display bioenergetic properties of their mitochondria. When *A. castellanii* cells shift from the exponential phase to the stationary phase, a decrease in the activity and protein level of AcAOX and AcUCP has previously been observed (Czarna et al. 2007).

Discussion

The addition of a moderate (1.4 mM) concentration of H_2O_2 to *A. castellanii* batch cultures at different phases of growth, i.e., during intensive cell division (early H_2O_2-treated cells) and during slowing cell division approaching the stationary phase preceding encystment (late H_2O_2-treated cells), caused a different response to oxidative stress. In the former case, the addition of H_2O_2 to exponentially growing cells significantly delayed growth of the *A. castellanii* batch culture, and after approximately 48 h, these cells restarted intensive division and reached a density of a late exponential phase instead of a stationary phase as observed in untreated control cells. Moreover, mitochondria isolated from early H_2O_2-treated cells revealed an increased activity of the energy-conserving (cytochrome pathway) and energy-dissipating (AcUCP and AcAOX) systems; they exhibited bioenergetic properties of the mitochondria from the late exponential phase (Czarna et al. 2007). Therefore, the 24 h regeneration period during which the cells underwent intensive division allowed the cells to overcome H_2O_2-induced oxidative stress. In mitochondria isolated from early H_2O_2-treated cells, no damage to their bioenergetic function and either no increase or a slight increase in oxidized proteins was observed.

In contrast to early H_2O_2-treated cells, addition of H_2O_2 to *A. castellanii* cells during slowing cell division (at the intermediary phase) did not considerably influence the growth conditions of the batch culture while seriously affecting mitochondrial bioenergetic function. Treated cells remained viable and maintained mitochondrial integrity. In late H_2O_2-treated cells, oxidative damage was revealed by significantly elevated levels of oxidized protein in the mitochondria. Moreover, in mitochondria isolated from these cells, the cytochrome pathway activity (uncoupled respiration), the efficiency of oxidative phosphorylation (ADP/O ratio), the membrane potential and the endogenous Q reduction level of the resting state were lowered. These results indicate that the components of the main mitochondrial respiratory pathway and likely ATP synthase in *A. castellanii* were damaged under H_2O_2-induced oxidative stress conditions. Similar results were observed in the mitochondria of plants treated with elevated H_2O_2 concentrations. Treatment of *Arabidopsis* cell cultures with 88 mM H_2O_2 for 7 days resulted in the degradation of the tricarboxylic acid cycle and some respiratory chain proteins as well as subunits of ATP synthase; these changes led to a decrease in respiration, but mitochondrial integrity was maintained (Sweetlove et al. 2002). In *A. castellanii* mitochondria from late H_2O_2-treated cells, an increase in the amount of membranous Q could allow a decreased Q pool reduction level (at a given influx of reducing substrates to the mitochondria) and consequently reduce production of harmful ROS. For the first time, this study has shown that the regulation of Q content in the inner mitochondrial membrane can play a role in the response to oxidative stress.

Because the action of AcUCP is driven by the membrane potential, which depends on the cytochrome pathway activity (Jarmuszkiewicz et al. 1999), it is not surprising that a significant decline either in the AcUCP activity and protein level was observed in mitochondria isolated from late H_2O_2-treated cells. Moreover, an increase in the amount of membranous Q and consequently a decrease in the Q reduction level (at a given influx of reducing substrates to the mitochondria) observed in the mitochondria from late H_2O_2-treated cells indicate that there is a low engagement of the present AcUCP. As shown previously, the inhibition efficiency of purine nucleotides for AcUCP-sustained uncoupling in *A. castellanii* mitochondria depends on the endogenous Q redox state, specifically on ubiquinol (QH_2) content (Jarmuszkiewicz et al. 2005b; Swida et al. 2008; Woyda-Ploszczyca and Jarmuszkiewicz 2011). Thus, in *A. castellanii* cells with a damaged mitochondrial cytochrome pathway, AcUCP is not involved in antioxidant defense as a response protein to oxidative stress.

In late *A. castellanii* H_2O_2-treated cells, H_2O_2-induced oxidative stress led to reduced respiratory capacity of the main cytochrome pathway but not the ubiquinol alternative

pathway (AcAOX), which was up-regulated and exhibited increased protein amounts and activity. This result is consistent with an attempt by the *A. castellanii* cells to maintain total respiratory capacity of the mitochondria in stressed cells compared to control cells. In contrast, in H_2O_2-treated *Arabidopsis* cells, oxidative stress reduces the mitochondrial respiratory capacity through both the cytochrome and AOX pathways, primarily by inhibiting electron flow through the respiratory dehydrogenases into the Q pool rather than by inhibiting the activity of respiratory oxidases (Sweetlove et al. 2002). Conversely, in *A. castellanii* mitochondria from late H_2O_2-treated cells, the rate of cyanide-resistant respiration (unstimulated and GMP-stimulated) was higher than that in mitochondria from control cells, and this effect was independent of the respiratory substrate used,. Unaffected respiratory dehydrogenase activities (at least the external NADH dehydrogenase activity) at concomitant lowered cytochrome pathway capacity establish conditions that cause increased demand for alternative pathway (AcAOX) activity. Our results indicate that in unicellular organisms, such as *A. castellanii*, AOX acts as a response protein to oxidative stress that allows increased mitochondrial oxygen consumption that could lead to some improvement in biosynthesis and growth under conditions of oxidative damage caused by H_2O_2. Increased AcAOX activity during oxidative stress could diminish the increase in reducing power, thus providing NAD^+ to the Krebs cycle and glycolysis and consequently providing carbon skeletons for biosynthesis. Furthermore, the increased AcAOX activity, which is accompanied by increased protein levels, would attempt to maintain the rate of mitochondrial electron transport to oxygen, compensating for the higher sensitivity of the cytochrome pathway to oxidative damage and thereby preventing production of harmful oxygen species. In *A. castellanii* mitochondria, AcAOX could also be directly involved in antioxidant defense because its activation lowers ROS formation (Czarna and Jarmuszkiewicz 2005). Protection against mitochondrial oxidative stress, involving maintenance of the balance of cell energy metabolism and the limitation of mitochondrial ROS production, may be a physiological role of AOX in unicellular organisms, especially when the secondary mitochondrial energy-dissipating systems (UCP) cannot work.

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