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Response of *Acanthamoeba castellanii* mitochondria to oxidative stress

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The purpose of this study was to examine the effects of oxidative stress caused by hydroperoxide (H_2O_2) in the presence of iron ions (Fe^{2+}) on mitochondria of the amoeba *Acanthamoeba castellanii*. We used isolated mitochondria of *A. castellanii* and exposed them to four levels of H_2O_2 concentration: 0.5, 5, 15, and 25 mM. We measured basic energetics of mitochondria: oxygen consumption in phosphorylation state (state 3) and resting state (state 4), respiratory coefficient rates (RC), ADP/O ratios, membrane potential ($\Delta\Psi_m$), ability to accumulate Ca^{2+}, and cytochrome *c* release. Our results show that the increasing concentrations of H_2O_2 stimulates respiration in states 3 and 4. The highest concentration of H_2O_2 caused a 3-fold increase in respiration in state 3 compared to the control. Respiratory coefficients and ADP/O ratios decreased with increasing stress conditions. Membrane potential significantly collapsed with increasing hydroperoxide concentration. The ability to accumulate Ca^{2+} also decreased with the increasing stress treatment. The lowest stress treatment (0.5 mM H_2O_2) significantly decreased oxygen consumption in state 3 and 4, RC, and membrane potential. The ADP/O ratio decreased significantly under 5 mM H_2O_2 treatment, while Ca^{2+} accumulation rate decreased significantly at 15 mM H_2O_2. We also observed cytochrome *c* release under increasing stress conditions. However, this release was not linear. These results indicate that as low as 0.5 mM H_2O_2 with Fe^{2+} damage the basic energetics of mitochondria of the unicellular eukaryotic organism *Acanthamoeba castellanii*.

Keywords: *Acanthamoeba castellanii*, mitochondria, hydroperoxide, oxidative stress

INTRODUCTION

Living cells produce non-destructive levels of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) *via* the respiratory chain in mitochondria (Fleury *et al*., 2002; Chen *et al*., 2003; Czarna & Jarmuszkiewicz, 2005) and during enzymatic reactions involving oxidoreductases and NAD(P)H (Li & Trush, 1998).

There are many different exogenic inducers of ROS, such as: UV irradiance and relatively high concentrations of copper (Cu^+) or iron (Fe^{2+}) ions in the cell. Fe^{2+} reacts with H_2O_2 in the Fenton reaction inducing a rise in hydroxyl radical ($^{\bullet}OH$) molecules, which is a very reactive oxygen species (Minotti & Aust, 1987; Fleury *et al*., 2002). The excess of ROS or lack of cell antioxidants (catalases, dismutases, peroxidases, glutathione etc.) contributes to an oxidative stress occurrence, which in turn leads to damage of mitochondrial DNA, proteins, and membranes, accelerates ageing, and in many cases leads to cell death (Papa & Skulachev, 1997).

In the present work we used mitochondria of the non-photosynthetic soil protozoan *Acanthamoeba castellanii* (Protista), which combines some features of mammalian, fungal and plant mitochondria. The occurrence of respiratory chain is a mammalian, fungal and plant characteristic, whereas the alternative oxidase (AOX) occurs in plants and some primitive fungi (Jarmuszkiewicz *et al*., 1997). This

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Abbreviations: ADP/O, number of ADP molecules converted to ATP while 2 electrons are carried from substrate *via* respiratory chain to 1 atom of oxygen resulting in reducing it to 1 molecule of water; AOX, alternative oxidase; MA, malate; RC, respiratory rate coefficient; ROS, reactive oxygen species; VDAC, voltage-dependent anion selective channel; $\Delta\Psi_m$, mitochondrial membrane potential.

makes the amoeba an attractive model organism. In addition, mitochondria of *A. castellanii* are able to actively accumulate calcium ions (Domka-Popek & Michejda, 1986). The influx of Ca^{2+} occurs through the outer membrane *via* voltage dependent anion selective channel (VDAC) and *via* calcium uniporter in the inner membrane (Bernardi, 1999; Gincel *et al*., 2001). Calcium ion accumulation leads to an increase of oxygen consumption in the resting state (state 4) and simultaneously decreases the mitochondrial membrane potential $\Delta\Psi_m$. The active accumulation of Ca^{2+} requires protonmotive force generated by respiratory chain (Domka-Popek & Michejda, 1986). Mitochondria of *A. castellanii* also contain a vast amount of catalase (Michejda *et al*., 1988).

Both mitochondrion swelling caused by over-accumulation of Ca^{2+} (Balzacq *et al*., 2002; Halestrap *et al*., 2002) and membrane lipid peroxidation caused by excess amounts of ROS trigger mitochondrial cytochrome *c* release and set off a destructive cascade of events (Stridh *et al*., 1998; Shimizu *et al*., 1999; 2000; Martinou *et al*., 2000).

The objective of the present work was to examine the effect of the Fenton reaction (its substrates and product) on: $\Delta\Psi_m$ generation, Ca^{2+} ion uptake and accumulation ability, oxygen consumption, and cytochrome *c* release in mitochondria of *Acanthamoeba castellanii*. Little is known about the direct effect of oxidative stress on mitochondria of lower eukaryotic organisms such as the amoeba *A. castellanii*. To fill this information gap we conducted a series of experiments using increasing concentrations of H_2O_2 in the presence of Fe^{2+} and isolated mitochondria of *Acanthamoeba castellanii*.

MATERIALS AND METHODS

Cell culture. Cells of *A. castellanii* (Neff strain) were cultured as described earlier by Jarmuszkiewicz *et al*. (1997). Trophozoites of the amoeba were collected between 60–72 h following inoculation at the middle exponential phase (at a density of 5 to 6×10^6 cells/ml).

Mitochondria isolation. The cells were separated from culture medium using centrifugation ($900 \times g$, 2 min). The pellet was suspended in isolation buffer A (pH = 7.4, 0.14 M sucrose, 10 mM Tris/Cl) and centrifuged at $900 \times g$ for 2 min. The pellet was suspended in isolation buffer B (pH = 7.8, 0.25 M sucrose, 10 mM Tris/Cl, 0.5 mM EGTA, 2% BSA (bovine serum albumin)) and homogenized using a hand homogenizer. Homogenized cells were sieved through antiseptic gauze and centrifuged for 5 min at $1650 \times g$. The supernatant was centrifuged at $11\,250 \times g$ for 15 min. The pellet was suspended in isolation buffer C (pH = 7.4, 0.25 M sucrose, 10 mM Tris/Cl, 0.5 mM EGTA, 0.2% BSA) and centrifuged at $11\,250 \times g$ for 15 min. The pellet was suspended in incubation buffer D (pH = 7.4, 0.25 M sucrose, 10 mM Tris/Cl) and centrifuged as above. Purified mitochondria were suspended in a small amount of the incubation buffer and kept on ice. The isolation procedure was performed at 0–4°C. Mitochondrial protein concentration was determined by the biuret method using BSA as a standard.

The oxidative stress model. Oxidative stress model conditions (concentrations of H_2O_2 and incubation time) were established experimentally. Samples containing 2.5–3 mg of mitochondrial protein were treated with H_2O_2 at final concentrations of 0.5, 5, 15, and 25 mM. To trigger the Fenton reaction a small volume of Fe^{2+} ($FeCl_2$ diluted in 50 mM NaCl, pH = 7.0) was added. Autoxidation of free Fe^{2+} at neutral pH and an inert medium like NaCl is extremely slow (Minotti & Aust, 1987). Incubations were performed for 1 and 5 min (for the latter data not shown) at 0–4°C. The reactions were stopped by 100-fold dilution with the reaction medium and the samples were centrifuged at $10\,000 \times g$ at 0°C for 20 s. The pellets containing stressed mitochondria were suspended gently in 100 µl of the incubation buffer and then used for the measurements of oxygen consumption, membrane potential, and calcium ion uptake. Supernatants were collected and used to estimate cytochrome *c* release.

Membrane potential. Membrane potential was monitored with a TPP^+-specific electrode at room temperature. Weinbach's buffer (pH = 7.4, 120 mM KCl, 20 mM Tris/Cl, 8 mM $MgCl_2$, 3 mM KH_2PO_4, 0.2% BSA) was used as an incubation medium. Individual samples contained 2.5–3 mg of mitochondrial proteins. Malate (MA) was added as the respiratory substrate (3 mM), then 130 µM ADP, and 0.1 µM FCCP at the end to dissipate mitochondrial potential. The electrode was calibrated before each measurement. The membrane potential was calculated according to Kamo's equation without any correction because we were interested only in the relative changes in $\Delta\Psi_m$.

Oxygen consumption. Mitochondrial respiration was measured polarographically at room temperature using a Clark-type electrode in 3 ml of Weinbach's buffer containing: 3 mM malate, 30 µM ADP = 90 nmols (as *pre puls*), 120 µM = 360 nmols ADP, and 1 µM FCCP, and with 2.5–3 mg of mitochondrial protein. We calculated the respiratory control coefficient (RC) and the ADP/O ratio for each measurement.

Calcium ion uptake. The measurements of Ca^{2+} uptake and accumulation by mitochondria were carried out using a TPP^+-specific electrode at room temperature for 2.5–3 mg mitochondrial protein per individual measurement. Weinbach's

buffer was used as medium with 3 mM malate as the respiration substrate. After malate addition, known amounts of calcium ions (about 150 nmols/mg of mitochondrial protein) were sequentially added to the medium until the membrane potential collapsed. Kamo's equation was used to calculate the membrane potential. Calcium ion portions were added and the membrane potential and time of $\Delta\Psi_m$ rebuilding were measured. Mitochondria disruption was assumed when no $\Delta\Psi_m$ was observed.

Cytochrome *c* release. Cytochrome *c* release was assessed spectrophotometrically at 550 nm. The measurements were monitored in quartz cuvettes containing 1 ml of mitochondrial solution with a small amount of sodium dithionite to reduce released cytochrome *c*. We used supernatants of stressed mitochondria after centrifugation and suspension in the incubation buffer (2.5–3 mg of mitochondrial protein per reaction were used).

Statistics. For all variables, statistical differences among treatments were analyzed using analysis of variance (GLM procedures). Multiple comparisons among traits for different stress variants were conducted using Tukey's HSD test. Relationships among the studied traits were determined using correlation and regression analyses. All statistical analyses were conducted with JMP software (version 5.0.1a, SAS Institute, Cary, NC, USA).

RESULTS

Respiration rates, respiration rate coefficients (RC) and ADP/O ratios

Our results indicate that increasing concentration of the Fenton reagent (H_2O_2) stimulates respiration in the phosphorylation state (state 3) and the resting state (state 4) (Fig. 1a, b). The effects were concentration-dependent (Fig. 1 a, b). Increasing stress conditions increased respiration in state 3 ($P < 0.0001$) and state 4 ($P < 0.0001$). The highest concentration of H_2O_2 increased respiration about 3-fold compared with the control in state 3 (Fig. 1a), and 12.5-fold in state 4 (Fig. 1b). Based on the respiration rates in states 3 and 4 we calculated respiration rate coefficients (RC) and ADP/O ratios for the stress and control treatments. Both RC and ADP/O decreased with increasing concentration of H_2O_2 ($P < 0.0001$, Fig. 2 and Fig. 3). The highest respiration coefficient was 11.35 ± 0.5 for the control, whereas the lowest was 2.86 ± 0.05 for the 25 mM H_2O_2 treatment (Fig. 2). The highest ADP/O value was for the control, 2.9 ± 0.02, whereas the lowest was 1.13 ± 0.01 for the 25 mM treatment (Fig. 3). Tukey's HSD test revealed no significant difference between the ADP/O ratios for the control and 0.5 mM stress treatment. However, the whole model was highly significant.

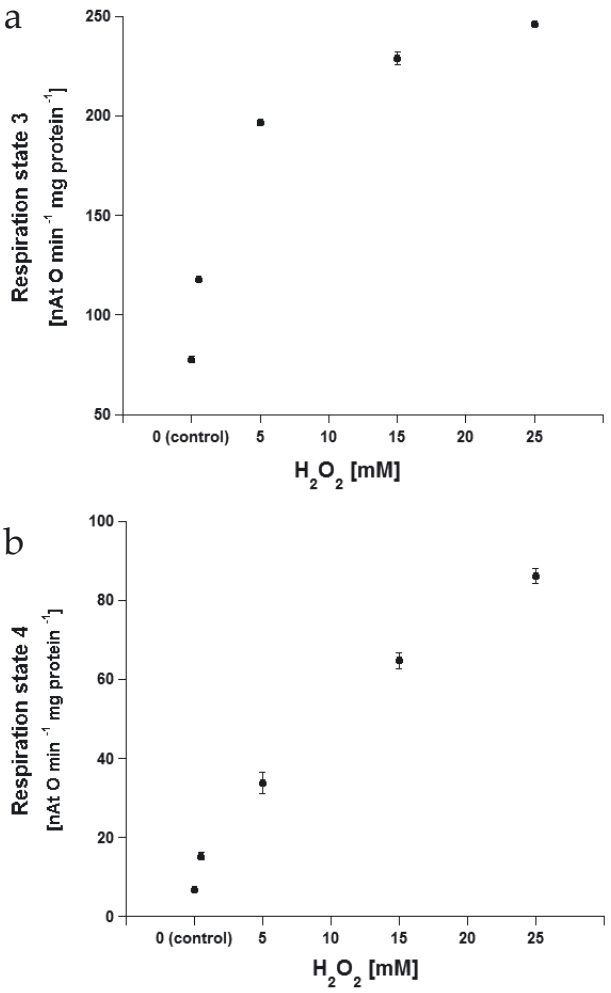

Figure 1. Respiration rates of *A. castellanii* mitochondria in states 3 (a) and 4 (b) for control and stress treatments. S.E. bars are shown. P-values derived from one-way ANOVA.

Mitochondrial membrane potential ΔΨm and active accumulation of Ca^{2+}

Mitochondrial membrane potential values generated after malate addition for all treatments are presented in Fig. 4. The rates gave us information about mitochondrial membrane coupling. The ability and timing of ADP transformation to ATP was calculated after addition of 360 nmols of ADP to the reaction medium. Membrane potential rates significantly decreased with increasing stress conditions from the highest (196.5 ± 0.58 mV) for the control to the lowest (159 ± 0.82 mV) for the 25 mM H_2O_2 stress treatment. The whole model was highly significant ($P < 0.0001$) and all variables were significant based on Tukey's HSD test (Fig. 4). The lowest stress conditions (0.5

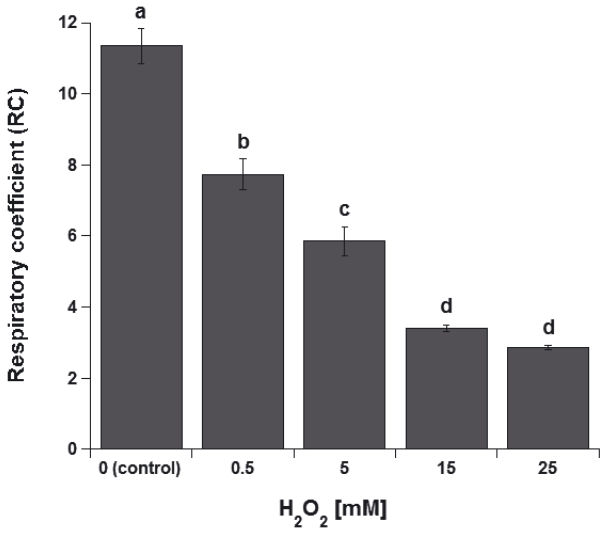

Figura 2. Respiratory coefficients (RC) of *A. castellanii* mitochondria for control and stress treatments (± S.E.).
For each variant, means with different letters are significantly different (Tukey's HSD test, $P = 0.05$). *P*-values are based on one-way ANOVA.

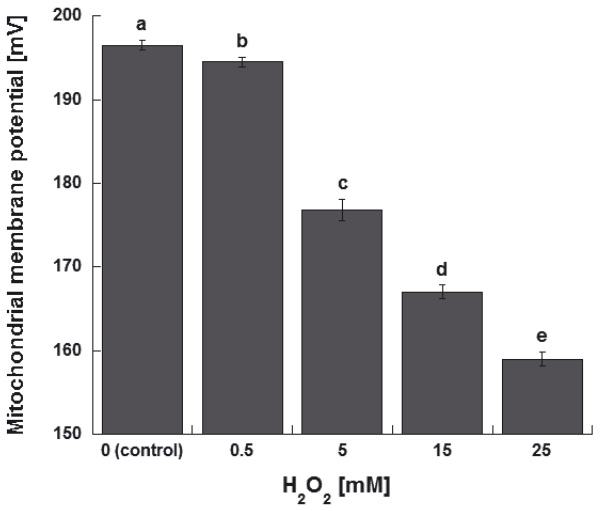

Figure 4. The mitochondrial membrane potential values of *A. castellanii* mitochondria generated after malate addition (± SE).
For each variant, means with different letters are significantly different (Tukey's HSD test, $P = 0.05$). *P*-values are based on one-way ANOVA.

mM H_2O_2) brought about a significant collapse of membrane potential in mitochondria of *A. castellanii* (Fig. 4). Active Ca^{2+} accumulation was measured as μmols of Ca^{2+} added to the reaction medium containing *A. castellanii* mitochondria (Fig. 5). The measurements were performed as long as the mitochondrial membrane potential was collapsed. The measurements made it possible to calculate Ca^{2+} accumulation rates expressed as the amount of Ca^{2+} (μmols) accumulated during one minute per one milligram of mitochondrial protein (Fig. 5). We calculated the rates for all treatments except for the highest stress condition (25 mM H_2O_2) because the time of $\Delta\Psi_m$ depletion was under 1 min and longer measurement could have given us a biased rate. The accumulation of calcium ions decreased with increasing stress conditions (Fig. 5). The whole model was statistically significant ($P < 0.0001$). However, there was no significant difference between the 0.5 mM and 5 mM stress treatments. Control mitochondria of *A. castellanii* accumulated on average 4.25 μmols (per 2.5–3 mg of mitochondrial protein) before depletion, whereas mitochondria incubated in 25 mM H_2O_2 accumulated less than 0.5 μmol of Ca^{2+} before depletion. The speed of accumulation of calcium ions varied among treatments (Fig. 5; $P < 0.0001$). To obtain relevant factor reflecting the ability of *A. castellanii* mitochondria to actively accumulate Ca^{2+} we used Ca^{2+} accumulation rates (Fig. 5). The rates decreased with increasing stress conditions. The highest was 0.16 μmol min^{-1} mg^{-1} for the control and the lowest was 0.08 μmol min^{-1} mg^{-1} for the 15 mM treatment. The whole model was highly significant: $P = 0.0019$. However, there was no significant difference among control, 0.5 mM and 5 mM treatments based on the Tukey's HSD test.

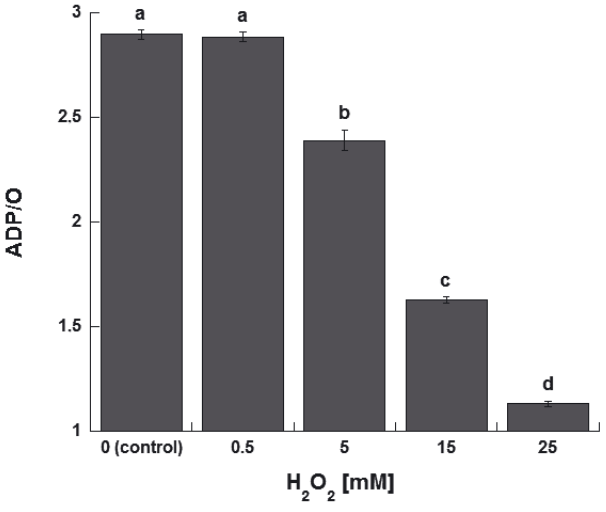

Figura 3. The ADP/O ratios of *A. castellanii* mitochondria for control and stress treatments (± S.E.).
For each variant, means with different letters are significantly different (Tukey's HSD test, $P = 0.05$). *P*-values are based on one-way ANOVA.

Cytochrome *c* release

To measure cytochrome *c* released from the intermembrane space as a result of outer membrane peroxidation, we conducted spectrophotometrical

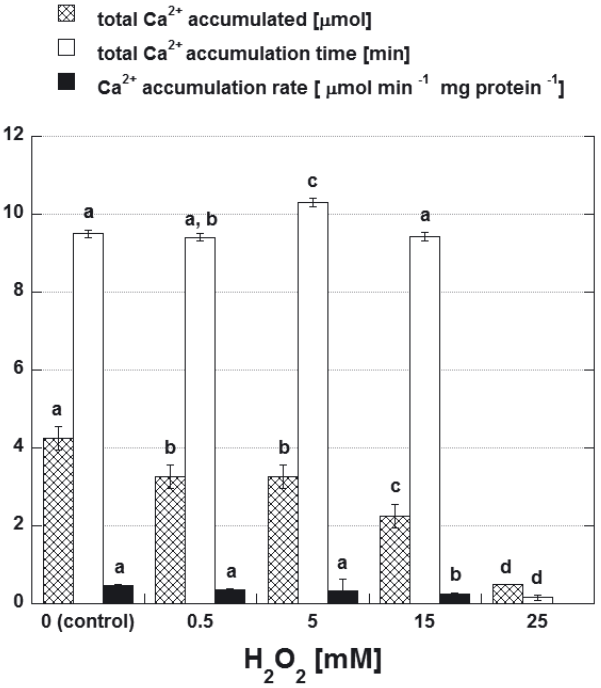

Figure 5. Calcium ion uptake by mitochondria of *A. castellanii*.
The amounts of Ca^{2+} accumulated by mitochondria of *A. castellanii*, estimated time of *A. castellanii* mitochondria over-accumulation and disruption, and Ca^{2+} accumulation rates are shown (± S.E.). For each variant, means with different letters are significantly different (Tukey's HSD test, $P = 0.05$). P-values are based on one-way ANOVA.

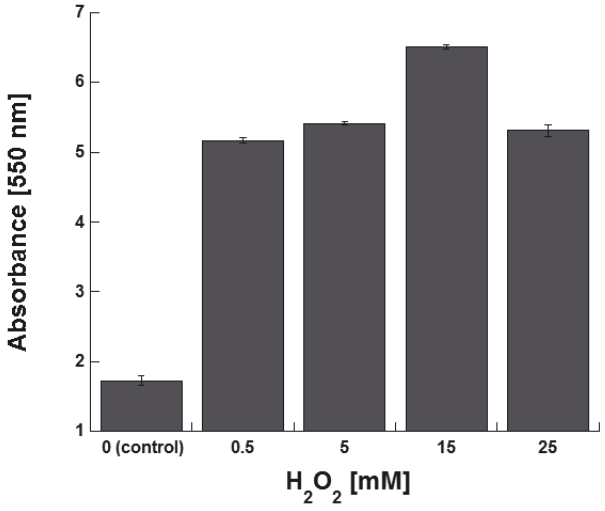

Figure 6. Release of cytochrome *c* from *A. castellanii* mitochondria assessed spectrophotometrically at the wavelength of 550 nm (± S.E.).

measurements at 550 nm of supernatants of mitochondria incubated with H_2O_2 and Fe^{2+}. For each measurement the same volume of supernatant was used. The absorbance values of released cytochrome *c* shown in Fig. 6 were means of four independent measurements. Surprisingly, we did not observe a consistent increase of cytochrome *c* release from mitochondria of *A. castellanii* subjected to increasing stress conditions. Instead, an increase was observed from the control to the 15 mM treatments, and a slight decrease for the 25 mM treatment compared to the 15 mM treatment. This result may suggest that a heme group in cytochrome *c* underwent peroxidation by the Fenton reagents and product during the incubation.

DISCUSSION

Reactive oxygen species are involved in cell damage and death. Intact cells that start to fail when oxidative stress occurs launch antioxidant defences to detoxify these harmful compounds (Radi *et al*., 1993; Lenaz *et al*., 1999; Fleury *et al*., 2002). However, ROS are not only dangerous molecules for the cell. They also display a physiological role as mediators in signal transduction pathways (Fleury *et al*., 2002). Hydrogen peroxide is a signalling molecule that controls a variety of stress responses and physiological adjustments in plants under stress conditions (Ślesak *et al*., 2007).

The results of our study revealed that the Fenton reaction (its reagents and product) triggers oxygen consumption increase, cytochrome *c* release, membrane potential dissipation, and loss of Ca^{2+} ion accumulation in mitochondria of *Acanthamoeba castellanii*.

Two parameters, RC and ADP/O, informed us which mitochondrial component, respiratory chain complexes or ATP-synthase, may have been initially damaged by oxidative stress. Damage to ATP synthase caused RC to decrease as a consequence of non-effective protonmotive force and a decline in ATP synthesis. The respiratory chain was still able to build the protonmotive force but the process required extra time. In this case, the ADP/O ratio for control and 0.5 mM stress treatments did not change significantly (Fig. 3). At the same time, RC decreased significantly for the 0.5 mM treatment in comparison with the control (Fig. 2). This occurred because the ADP/O ratio depends on proton influx (through the respiratory chain pumps) and efflux (by ATP synthase) stoichiometry. When respiratory chain complexes are damaged, the ADP/O ratio changes as a result of proton stoichiometry disruption.

Notably, our experiments demonstrate that H_2O_2 concentration as low as 0.5 mM in the presence of Fe^{2+} contributes to disturbances in the generation of membrane potential (Fig. 4), transport of electrons through the respiratory chain, oxidative phosphorylation (not shown), and uptake and accumulation of Ca^{2+} (Fig. 5). The respiration rate coefficient de-

creased by 32% after one minute of incubation with 0.5 mM H_2O_2 (Fig. 2), while the ADP/O did not change significantly (Fig. 3). Under these conditions, Ca^{2+} active accumulation ability also decreased by 1000 nmols in comparison with the control (Fig. 5). Therefore, it is possible that even 0.5 mM H_2O_2 may, within a short time, break outer membrane integrity and/or ATP synthase complexes. In addition, this stress condition led to the release of cytochrome *c* (Fig. 6), which is in agreement with results of prior studies (Minotti & Aust, 1987; Kowaltowski *et al*., 1996; Kroemer *et al*., 1997; Martinou *et al*., 2000). The following increase of oxidative stress conditions contributed to the decrease both RC and ADP/O and ability to accumulate calcium ions (Figs. 2, 3, and 5, respectively). These effects may be a result of membrane peroxidation and, in consequence, membrane potential depletion and lack of the ability to accumulate Ca^{2+}. Reactive oxygen species damaged respiratory chain complexes, which in turn disrupted proper ADP/O stoichiometry.

Calcium ions are transported through the outer membrane by the *voltage dependent anion selective channel* (VDAC) that contains Ca^{2+}-binding domains (Gincel *et al*., 2001) and/or by $\Delta\Psi_m$-dependent Ca^{2+} uniporter located in the inner mitochondrial membrane (Bernardi, 1999). The results of our study suggest that the loss of the ability to accumulate Ca^{2+} is not a result of $\Delta\Psi_m$ depletion alone. Reactive oxygen species may also damage both calcium pathway proteins (VDAC and Ca^{2+} uniporter). The intracellular Ca^{2+} concentration stimulates Ca^{2+} uniport channel activity. The Ca^{2+} uniporter contains a regulatory Mg^{2+}-binding domain. Binding of Mg^{2+} by the Ca^{2+} uniporter influences the kinetics of Ca^{2+} ion flux, while Mg^{2+} are not channeled by the Ca^{2+} uniporter (Bernardi, 1999). The putative groups binding Mg^{2+} are sulphydryl groups of cysteine (Wudarczyk *et al*., 1999). Reactive oxygen species may decrease Ca^{2+} uniporter activity by oxidizing these sulphydryl groups and therefore reducing Mg^{2+} binding. Reactive oxygen species readily penetrate lipid bilayers and damage cell proteins. The question arising is: which channel system allows Ca^{2+} to get through the outer mitochondrial membrane and how do free radicals damage the channel system in *A. castellanii*? Previous studies show that VDAC contains Ca^{2+}-binding domains in rat liver mitochondria (Gincel *et al*., 2001). They point to a paper concerning similar experiments conducted on *Paramecium aurelia* (Protista), in which VDAC did not display a Ca^{2+} affinity (Schein *et al*., 1976).

In higher plants, H_2O_2 molecules produced by mitochondria may play a secondary signal role by inducing *AOX* gene(s) or other genes related to resistance mechanisms after respiratory chain damage. Vyssokikh and Brdiczka (2003) observed increased respiration and *AOX1* gene expression in the case of respiratory chain inhibition. In our study, we also noted increased respiration following increased oxidative stress conditions (Figs. 1a and b). Experiments on *A. castellanii* cells that show a higher AOX protein content during the logarithmic growth phase would be necessary to determine whether this alternative pathway actually raises the probability of survival under stress conditions (Purvis, 1997; Popov *et al*., 1997).

The lowest H_2O_2 concentration used in our study was 5-fold higher than that used for ferrous and hydrogen peroxide-dependent peroxidation of phospholipid liposomes (Minotti & Aust, 1987). This suggests that mitochondria of *A. castellanii* contain an effective antioxidant-defence system for scavenging ROS and preventing damage. One of the system component might be a catalase, which occurs at a high concentration in *A. castellanii* mitochondria (Michejda *et al*., 1988). Another is alternative oxidase, which is also present in plant mitochondria. Czarna & Jarmuszkiewicz (2005) proved that activation of AOX lowers ROS production in mitochondria of *A. castellanii*. The study used transgenic cultured tobacco cells with altered levels of AOX also indicated that the alternative pathway in plant mitochondria functions as a mechanism to decrease the formation of reactive oxygen species produced during respiratory electron transport (Maxwell *et al*., 1999). Sweetlove *et al*. (2002) demonstrated that treatment of *Arabidopsis* cell cultures with 88 mM H_2O_2 resulted in changes in isolated mitochondria. The authors observed a degradation of the tricarboxylic acid (TCA) cycle and respiratory chain proteins, and, contrary to our results, a decrease in respiration. Their results indicate that oxidative stress reduces the respiratory capacity primarily by inhibiting electron flow through respiratory dehydrogenases into the Q pool and by reducing the activity of the TCA cycle, rather than by affecting the activity of respiratory oxidases. The increase of oxygen consumption in our study may be due to an electron efflux *via* respiratory dehydrogenase into the Q pool and interception by AOX molecules. Dissipation of membrane potential, RC, and ADP/O suggest a loss of integrity of outer membrane that prevented conservation of the proton electrochemical gradient while increasing the consumption of oxygen.

Tiwari *et al*. (2002) observed faster oxygen consumption in mitochondria isolated from *Arabidopsis* cells treated with 1 mM or 5 mM H_2O_2. However, the authors explained that the differences in electron transport were not associated with alternative oxidase activity because oxygen consumption was completely blocked by the addition of potassium cyanide.

In conclusion, this study allowed us to observe direct effects of reactive oxygen species on

mitochondria of the soil protozoan *Acanthamoeba castellanii*. As low as 0.5 mM concentration of H_2O_2 in the presence of Fe^{2+} damaged the energetics of mitochondria of *A. castellanii*. We observed increased respiration in state 3 and 4, decrease of $\Delta\Psi_{m'}$ respiratory rate coefficient (RC), ADP/O ratio, Ca^{2+} uptake, and an increased release of cytochrome *c* in mitochondria of *A. castellanii* subjected to oxidative stress.

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