Redox Biology 18 (2018) 256–265

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level

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ARTICLE INFO

Keywords:
Mitochondria
Acanthamoeba castellanii
Reactive oxygen species
Coenzyme Q reduction level

ABSTRACT

Our aim was to elucidate the relationship between the rate of mitochondrial reactive oxygen species (mROS) formation and the reduction level of the mitochondrial coenzyme Q (mQ) pool under various levels of engagement of the mQ-reducing pathway (succinate dehydrogenase, complex II) and mQH_2-oxidizing pathways (the cytochrome pathway and alternative oxidase pathway, (AOX)) in mitochondria isolated from the amoeba *Acanthamoeba castellanii*. The mQ pool was shifted to a more reduced state by inhibition of mQH_2-oxidizing pathways (complex III and complex IV of the cytochrome pathway, and AOX) and the oxidative phosphorylation system. The mQ reduction level was lowered by decreasing the electron supply from succinate dehydrogenase and by stimulating the activity of the cytochrome or AOX pathways. The results indicate a direct dependence of mROS formation on the reduction level of the mQ pool for both mQH_2-oxidizing pathways. A higher mQ reduction level leads to a higher mROS formation. For the cytochrome pathway, mROS generation depends nonlinearly upon the mQ reduction level, with a stronger dependency observed at values higher than the mQ reduction level of the phosphorylating state (~ 35%). AOX becomes more engaged at higher mQ pool reduction levels (above 40%), when mROS production via the cytochrome pathway increases. We propose that the mQ pool reduction level (endogenous mQ redox state) could be a useful endogenous reporter that allows indirect assessment of overall mROS production in mitochondria.

1. Introduction

Mitochondrial coenzyme Q (mQ, ubiquinone) is an essential electron carrier that plays a central role in the mitochondrial electron transport respiratory chain [1,2]. Mitochondrial Q shuttles electrons between dehydrogenases and the oxidizing pathway(s) of the mitochondrial respiratory chain and is also involved in the formation of superoxide from semiubiquinone radicals by the respiratory chain, which can lead to mitochondrial oxidative damage [3–5]. It is widely accepted that mitochondrial reactive oxygen species (mROS) production depends on the level of reduction of mitochondrial electron carriers, especially on mQ. Surprisingly, there are only few data relating mQH_2/Q ratio [6] or mQ reduction level [7] with mROS generation.

The amoeba *Acanthamoeba castellanii* is a nonphotosynthesizing free-living protozoan belonging to the group that diverged from the animal/fungal line after the split from plants [8]. As an opportunistic pathogen that can cause serious diseases in humans, *A. castellanii* is an evolutionarily and medically important amoebozoan. This species presents features, including mitochondrial physiology, that are common to plants, fungi and animals. In addition to several dehydrogenases, the plant-type respiratory chain of *A. castellanii* mitochondria contains two ubiquinol (mQH_2)-oxidizing pathways, namely, the classical antimycin A- and cyanide-sensitive cytochrome pathway and the alternative benzohydroxamate- and propyl gallate-sensitive ubiquinol oxidase (AOX) pathway [9–11]. The mQ pool plays a central role in the respiratory chain; respiratory substrate-oxidizing dehydrogenases reduce mQ to mQH_2, and the two oxidizing pathways convert mQH_2 to mQ. Electron transfer via the AOX pathway does not result in proton pumping and is therefore not coupled to the mitochondrial production of ATP. The study of mitochondrial respiration of succinate (complex II substrate) in *A. castellanii* allows investigation of the kinetics of two mQH_2-oxidizing pathways; one proton electrochemical gradient

Abbreviations: AOX, alternative oxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; mROS, mitochondrial reactive oxygen species; mQ, mitochondrial coenzyme Q, ubiquinone; mQox, oxidized Q; mQH_2, reduced Q (ubiquinol); mQtot, total pool of endogenous Q in the inner mitochondrial membrane; mQH_2/mQtot, reduction level of Q; $m\Delta\Psi$, mitochondrial membrane electric potential; OXPHOS, oxidative phosphorylation; $\Delta\mu H^+$, proton electrochemical gradient

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https://doi.org/10.1016/j.redox.2018.07.018
Received 9 July 2018; Received in revised form 19 July 2018; Accepted 20 July 2018
Available online 23 July 2018

($\Delta\mu H^+$)-generating pathway, consisting of the two proton-pumping complexes III and IV (the cytochrome pathway), and one $\Delta\mu H^+$-independent pathway (the AOX pathway). Because mROS production depends on $\Delta\mu H^+$ [4], investigation of *A. castellanii* mitochondria enables the determination of the relationship between mROS formation and the mQ reduction level at different mitochondrial membrane potential ($m\Delta\Psi$) values depending on the engagement of the two mQH_2-oxidizing pathways.

The aim of our work was to elucidate the relationship between mROS formation and the reduction level of the mQ pool under a variety of mitochondrial respiration conditions, i.e., at varying degrees of engagement of mQ-reducing the pathway (succinate dehydrogenase, complex II) and mQH_2-oxidizing pathways (the cytochrome pathway and AOX) in isolated *A. castellanii* mitochondria. The mQ reduction level was increased by decreasing electron flow out of the mQ pool via inhibition of the mQH_2-oxidizing pathways (complex III, complex IV, or AOX) or inhibition of the oxidative phosphorylation (OXPHOS) system (ATP synthase or ATP/ADP antiporter). The mQ pool was shifted to a more oxidized state by decreasing the electron supply from complex II via inhibition of the mQ-reducing pathway (substrate dehydrogenase) or by stimulation of the activities of the mQH_2-oxidizing pathways under uncoupling conditions (the cytochrome pathway) or under GMP activation (AOX). We measured the mQ reduction level under given mitochondrial oxygen consumption and mitochondrial membrane potential ($m\Delta\Psi$) conditions in terms of H_2O_2 formation.

2. Materials and methods

2.1. Acanthamoeba castellanii cell culture and isolation of mitochondria

Trophozoites of the *A. castellanii* strain Neff (ATCC®30010TM) were cultured as described previously [10]. Cells from 72-h cultures were inoculated (time 0) to a final density of approximately $2.5 \pm 0.4 \times 10^5$ cells $\times$ ml^{-1}. After approximately 40 h of exponential growth with a generation time (cell doubling time) of 8 h, the amoeba cultures reached the intermediate growth phase and then the stationary phase, the latter preceding transformation into cysts within a few hours. In this study, trophozoites of *A. castellanii* were harvested 48 h after inoculation, in the intermediate phase ($6.8 \pm 0.5 \times 10^6$ cells $\times$ ml^{-1}). Mitochondria were isolated in an isolation medium containing 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), 0.5 mM EGTA, and 0.2% bovine serum albumin (BSA) and then purified on a self-generating Percoll gradient (28%) for 45 min at 40,000 *g* [9]. Purified mitochondria were washed in isolation medium without BSA and EGTA. Protein concentrations of the isolated mitochondria were determined using the biuret method.

2.2. General measurement conditions

All measurements were performed in a standard incubation medium (28 °C) containing: 120 mM KCl, 20 mM Tris/HCl (pH 7.4), 3 mM KH_2PO_4, 8 mM $MgCl_2$, 1 mM EGTA, and 0.2% BSA with continuous stirring. Mitochondria (0.33 mg of protein/ml) were incubated with succinate (5 mM) as an oxidizable substrate of complex II in the presence of rotenone (2 μM) to block electron input from complex I. Respiratory rate, $m\Delta\Psi$, mQ reduction levels, and mROS formation were measured under (i) resting (nonphosphorylating, State 4) conditions, i.e., in the absence of exogenous ADP, and (ii) phosphorylating (State 3) conditions, i.e., in the presence of 1–2 mM ADP. The mQ-reducing pathway was titrated with increasing concentrations of malonate (an inhibitor of succinate dehydrogenase, complex II). The activity of a major mQH_2-oxidizing pathway (the cytochrome pathway) was varied with increasing concentrations of (i) antimycin A or cyanide (inhibitors of complex III and complex IV of the cytochrome pathway, respectively), (ii) oligomycin or carboxyatractyloside (inhibitors of ATP synthase and ATP/ADP antiporter, respectively), or (iii) uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) in the presence of AOX inhibitor. Parameters of the cytochrome pathway were measured in the presence of benzohydroxamate (1 mM) (respiratory rate, $m\Delta\Psi$, and mQ reduction level) or propyl gallate (3 μM) (H_2O_2 formation). The activity of the alternative mQH_2-oxidizing pathway (AOX) was varied with increasing concentrations of GMP (an allosteric activator of AOX, [12,13]) in the presence of 0.65 mM cyanide or 90 nM antimycin A (to exclude the activity of the cytochrome pathway). The relationships between the studied respiratory chain parameters (respiratory rate, $m\Delta\Psi$, mQ reduction levels, and H_2O_2 formation) and increasing concentrations of the respiratory chain and OXPHOS system modulators are shown in the Supplementary materials (Figs. S1–S6).

2.3. Mitochondrial oxygen consumption and mΔΨ measurements

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) in 3.0 ml of incubation medium (28 °C) with 1 mg of mitochondrial protein. Only high quality *A. castellanii* mitochondrial preparations, i.e., those with ADP/O values of ~1.40 (with succinate as a respiratory substrate) and respiratory control ratios of ~3.5, were used in all the experiments. For OXPHOS control, phosphorylating respiration (State 3) was measured after an ADP pre-pulse (50 μM) using 150 μM ADP as the main pulse. The total amount of oxygen consumed during phosphorylating respiration was used to calculate the ADP/O ratio. The $m\Delta\Psi$ measurements allowed fine control of the duration of phosphorylating respiration. Values of O_2 uptake are given in nmol O_2 $\times$ min^{-1} $\times$ mg $protein^{-1}$.

The $m\Delta\Psi$ was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode as described previously [14–16]. The TPP^+-specific electrode was calibrated with three sequential additions (0.8, 0.8, and 1.6 μM) of TPP^+. After each run, 0.5 μM FCCP was added to release TPP^+ for baseline correction. For calculation of the $\Delta\Psi$ value, the matrix volume of the amoeba mitochondria was assumed to be 2.0 μl $\times$ mg^{-1} protein. The calculation assumes that the TPP^+ distribution between the mitochondria and medium followed the Nernst equation. The values of $\Delta\Psi$ are given in mV.

2.4. Assay of H_2O_2 production by isolated mitochondria

The mitochondrial H_2O_2 production rate was measured by the Amplex Red-horseradish peroxidase method (Invitrogen) [17]. Horseradish peroxidase (0.1 U $\times$ ml^{-1}) catalyzes the H_2O_2-dependent oxidation of nonfluorescent Amplex Red (5 μM) to fluorescent resorufin red. Fluorescence was kinetically followed for 10 min at 545 nm (excitation), 590 nm (emission), and gain 150 using an Infinite M200 PRO Tecan multimode reader with 24-well plates. Mitochondria (0.17 mg of mitochondrial protein) were incubated in 0.5 ml of the standard incubation medium (see above) with 5 mM succinate as an oxidizable substrate in the presence of rotenone (2 μM). Because, at high concentrations, AOX inhibitors, benzohydroxamate and propyl gallate, are known to inhibit horseradish peroxidase activity and scavenge free radicals [18–21], H_2O_2 formation related to the cytochrome pathway activity was measured in the presence of a low concentration of propyl gallate (1.5 μM), which completely inhibits AOX activity. Because cyanide inhibits horseradish peroxidase activity [22], the H_2O_2 formation rate related to AOX activity was measured in the presence of 90 nM antimycin A to inhibit cytochrome pathway activity. Therefore, titration of H_2O_2 formation related to cytochrome pathway activity was performed only in the presence of increasing concentrations of antimycin A not cyanide. Reactions were monitored with constant stirring at 28 °C and calibrated with known amounts of H_2O_2 in the absence or presence of 1.5 μM propyl gallate. H_2O_2 production rates were determined from slopes calculated from readings obtained from several repeated 10-min measurements. Values of H_2O_2 production are given in