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Electrochemical study of hydrogen peroxide formation in isolated mitochondria

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ABSTRACT

Mitochondrial respiration generates reactive oxygen species that are involved in physiological and pathological processes.
The majority of methods, with exception of electron paramagnetic resonance, used to evaluate the identity, the rate and the conditions of the reactive oxygen species produced by mitochondria, are mainly based on oxidation sensitive markers. Following latest electrochemical methodology, we implemented a novel electrochemical assay for the investigation of aerobic metabolism in preparations of isolated mitochondria through simultaneous measurement of O_2 consumption and reactive species production. This electrochemical assay reveals active H_2O_2 production by respiring mouse liver mitochondria, and shows that ATP synthase activation and moderate depolarization increase the rate of H_2O_2 formation, suggesting that ATP synthesizing (state 3) mitochondria might contribute to oxidative stress or signaling.

1. Introduction

Incomplete reduction of O_2 during mitochondrial electron transfer chain (ETC) reactions generates superoxide anion, $O_2^{\bullet -}$ that is dismutated by SODs into hydrogen peroxide, H_2O_2. H_2O_2 is either degraded inside mitochondria by glutathione peroxidases, peroxiredoxins and catalases or can diffuse outside mitochondria being removed by extramitochondrial scavenging reactions [1,2].

Despite their toxicity, accumulating evidence support a physiological role for reactive oxygen species (ROS) (including: $O_2^{\bullet -}$, H_2O_2, and hydroxyl radical, OH•) in a variety of cellular processes, such as growth factors/hormones/cytokines intracellular signaling, genes expression regulation and programmed cell death (apoptosis) [3,4]. Among ROS, H_2O_2 is the best suited to function as a signaling agent since it is diffusible, less reactive and longer-lived than, for instance, $O_2^{\bullet -}$ and OH•, and it is therefore involved in several transduction pathways [5]. Since the rate of mitochondrial ROS formation is modulated, roughly speaking, by O_2 availability, substrate supply and ATP synthesis [6,7], the emerging picture is that mitochondria generate ROS in a regulated manner, such as to reflect the metabolic activity of the cell and/or to act as an oxygen sensor involved in the transduction of hypoxic signals [8,9]. Nevertheless, assessing the rate of mitochondrial ROS, and in particular H_2O_2 production, is a complicated issue due in part to inherent difficulties of available technologies for measuring these molecules. Except for electron paramagnetic spin resonance that can directly detect free radicals with unpaired electrons [10], all other methods for ROS detection are indirect. They measure either the reaction product between ROS and various probes or the fingerprints of oxidative stress on different endogenous molecules (DNA, lipids, proteins, low-molecular-mass antioxidants) [11]. In addition, most traditional assays for H_2O_2 measurement relay on fluorescent probes that often lack specificity and may result in artefactual signals [12–14].

In the search for other assays to measure ROS, electrochemistry may represent a valid alternative. Oxygen and its reactive species are particularly suited for these kinds of measurements since they can easily exchange electrons at an appropriate potential. Recently, reactive species produced by living cells in response to mechanical challenges have been measured directly and selectively through electrochemistry at platinized carbon fiber microelectrodes (PCE) [15,16].

To overcome the limitations of current methodologies for mitochondrial H_2O_2 measurement, we implemented an electrochemical assay for the simultaneous evaluation of O_2 consumption metabolism and H_2O_2 production in suspensions of isolated mitochondria. Here we show the results obtained with this approach on mouse liver mitochondria preparations in the presence of different energetic substrates, respiratory inducers and inhibitors.

Abbreviations: ETC, electron transfer chain; ROS, reactive oxygen species; SOD, superoxide dismutase; PCE, platinized carbon fiber microelectrodes; RNS, reactive nitrogen species; FCCP, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone.

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2. Material and methods

2.1. Preparation of isolated mitochondria

Mitochondria were purified from mouse liver through standard differential centrifugation method. 129Sv 3–6 months old male mice were killed by cervical dislocation, the liver was rapidly explanted and homogenized in ice-cold MTC isolation buffer (250 mM sucrose, 10 mM Tris HCl, 1 mM EGTA, pH 7.4). The liver homogenate was centrifuged for 10 min at 600 *g* for the removal of nuclei and cell debris and the supernatant centrifuged at 7000 *g* for 10 min. The mitochondria containing pellet was washed twice and finally resuspended in a minimal volume of MTC isolation buffer. All steps were performed at 4 °C. Protein concentration was measured using the biuret method. Experiments were performed in MTC assay buffer (125 mM KCl, 10 mM MOPS-Tris, 1 mM inorganic phosphate, 100 μM EGTA, pH 7.4).

2.2. Instrumentation for electrochemical measurements of O_2 metabolism

Simultaneous measurements of O_2 consumption and H_2O_2 production by isolated mitochondria were achieved integrating an oxygraph apparatus (Hansatech Instruments, Norfolk, England) with a system for the simultaneous detection of H_2O_2 (Fig. 1A). The electrochemical measurement of O_2 was performed at the Clark type electrode by reducing O_2 at a reduction potential of −0.7 V vs. Ag/AgCl reference electrode. For H_2O_2 detection, the chamber of the oxygraph was adapted in order to allocate a 10 μm PCE, an Ag/AgCl reference electrode and a Pt counter electrode. Electrochemical detection of H_2O_2 was performed by oxidizing this molecule at an oxidation potential of 0.6 V vs. Ag/AgCl reference electrode provided by a bipotentiostat (CH Instruments, Austin, TX). The choice of using 0.6 V to detect H_2O_2 was based on the fact that, although the plateau for H_2O_2 oxidation was reached at 0.4 V in PBS solution [17], in the presence of the mitochondrial suspension we observed a maximal current response for H_2O_2 at 0.6 V vs. Ag/AgCl (Supplementary Fig. 1). The two electrochemical cells for O_2 and, respectively, H_2O_2 detection were separated through a polytetrafluoroethylene (PTFE) membrane covering the Clark type electrode that assures the electrical insulation between the electrolytic solution above the Clark type electrode and the oxygraph chamber solution. The selectivity of the $[O_2]$ measurement, at Clark type electrode, is ensured by this design of the analytical: the Clark type electrode is separated in fact from the mitochondrial suspension by the O_2 permeable and O_2 selective PTFE membrane. For each measurement, we tested the integrity and impermeability of the PTFE shield verifying that the two electrochemical cells (one including the Clark type electrode and the other including the platinized carbon fiber/Pt CE / Ag/AgCl QRE) were isolated electrically. The reaction mixture was stirred by a magnetic follower that abolished local gradients of O_2 and H_2O_2. The chamber was isolated from the external environment through an O_2 impermeable plastic cap, without gas phase left between the assay solution and the cap.

For the preparation of the PCE, briefly: carbon fibers (Cytec Carbon Fibers, Greenville) were aspired inside borosilicate glass capillaries (Clark Electromedical Instruments, 1.2 mm O.D.×0.7 mm I.D.) and pulled with a micropipette puller (P-97, Sutter Instruments, U.S.A.) into two electrodes. The carbon fiber protruding from the glass was insulated by electrochemical deposition of a poly (oxy-phenylene) polymer. The surface of the tip was polished by grinding on 0.3 μm alumina paper and then platinized by reducing hydrogen hexachloroplatinate in the presence of lead acetate [15]. Each measurement was performed using a single PCE that was calibrated at the end with known concentrations of H_2O_2. Before measurements, the electrodes were left to equilibrate for 30 min in MTC assay buffer.

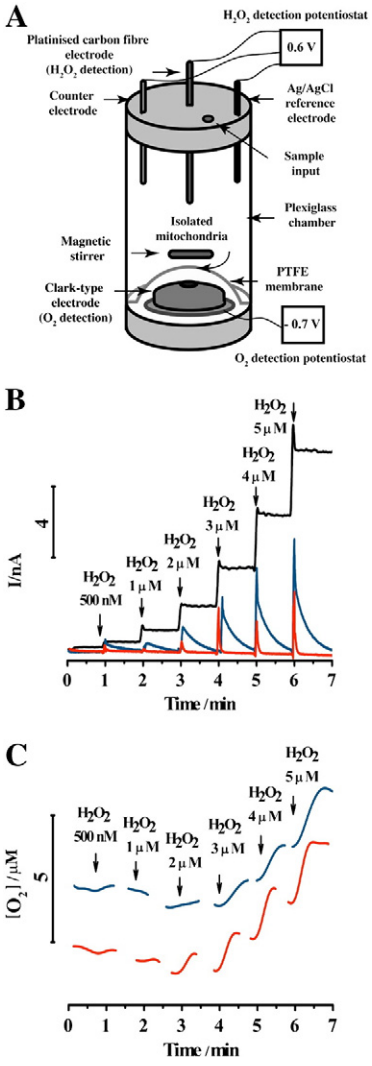

Fig. 1. Electrochemical instrumentation for simultaneous measurement of H_2O_2 and O_2 A. Scheme of the apparatus used for the simultaneous detection of H_2O_2 and O_2. B. Amperometric response of successive addition of H_2O_2 at PCE. A 0.6 V vs. Ag/AgCl potential was applied at PCE to oxidize H_2O_2. Measurements were performed in absence of catalase (black line), in presence of 20 nM catalase (blue line) and 200 nM catalase (red line). C. Simultaneous detection of O_2 concentration (in the same chamber as B) by Clark type electrode. A −0.7 vs. Ag/AgCl potential was applied to Clark electrode to reduce O_2. The measurements were performed in 20 nM catalase (blue line), and 200 nM catalase (red line). The amperometric current values are converted in O_2 concentration in the present plot. Where indicated by the arrows, known concentrations of H_2O_2 (0.5–5 μM) were added.

2.3. Data analysis for amperometric measurements of H_2O_2

Once a potential has been applied, PCEs undergo an equilibration phase due to diffusion effects, slow partial oxidation, and modification of the active sites of the platinized surface [18]. The outcome of these equilibration processes is a progressive and continuous reduction of the oxidation current registered at the electrode. The equilibration curve thus generated can be described by an exponential function (Eq. (1)) that we have used to extrapolate the baseline:

$$y = y_0 + A_1 \times (1-\exp(-x/t_1)) + A_2 \times (1-\exp(-x/t_2)) \quad (1)$$

A_1, A_2, t_1, and t_2 are dimensionless parameters, specific for every curve, that are determined through the fitting procedure. Thus, any specific electrical event can be revealed by subtracting the baseline from