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Measuring mitochondrial reactive oxygen species

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Abstract

This article examines recent methods for measuring reactive oxygen species produced in isolated mitochondria and within live cells, with particular emphasis on the detection of hydrogen peroxide. Protocols for reliable measurements of mitochondrial hydrogen peroxide are presented, while the advantages and pitfalls of these and other methods are discussed. New developments in the detection of lipid peroxidation are outlined. Advice is also provided to aid the interpretation of cellular data with respect to the contribution of oxygen radical production by different components of the mitochondrial respiratory chain.

1. Introduction

Mitochondria are major generators of reactive oxygen species (ROS) in cells and tissues [1–3]. ROS include, predominantly, the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet-}$). Of these, H_2O_2 is the most stable and abundant, also because it is the by-product of superoxide scavenging by the superoxide dismutase (SOD) enzymes. Nitroxy radicals also contribute to mitochondrial ROS, especially under certain conditions [1,4]. Here, I focus on methods for investigating the mitochondrial production of H_2O_2 and discuss new developments in the measurement of lipid peroxidation; see Turrens [2] for a review of other methods to detect mitochondrial ROS, and Cadenas et al. [4] for a recent review on nitroxy radicals and mitochondria.

In general, measurements of mitochondrial ROS production can be carried out either in vitro, using conventional preparations of isolated mitochondria, or in live cells. Unfortunately, several of the methods originally applied to detect and investigate mitochondrial ROS are not suitable for cellular studies [5]. Measuring mitochondrial ROS is complicated not only by methodological problems, but also by common views on the relative contribution of individual redox groups that may lead to incorrect interpretations of data. On reading recent literature regarding ROS and mitochondria one easily finds in vitro data that are inconsistent with, or contradict, cellular data. It is one purpose of this article to rectify views that can complicate or mislead the interpretation of data on mitochondrial radicals produced in living cells.

2. Methods for ROS detection with isolated mitochondrial preparations in vitro

2.1. Superoxide

Superoxide is the first species to be produced by autoxidation of mitochondrial redox groups such as iron–sulfur clusters and semiquinone radicals [1–3]. Due to the spontaneous and enzyme-driven dismutation of the superoxide radical, as well as its reactivity with other compounds, its detection is generally more arduous than that of hydrogen peroxide. The early methods for the measurement of superoxide production with mitochondrial preparations have been reviewed [2,3]. Here I mention two procedures that have been introduced in the last few years and are generally much more sensitive than previous spectrophotometric methods. The first procedure uses chemical analogs of the *Cyprinida* luciferin such as 2-methyl-6-phenyl-3,7-dinitroimidazo[1,2-*a*]pyrazin-3-one (CLA) [6] and its

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methoxy derivative (MCLA) [7], which produce a chemiluminescent adduct after reaction with superoxide. Despite the potential high sensitivity of chemiluminescence detection, the applications of this method are limited by the relatively high background reactions with mitochondria and the strong inhibition of complex I activity induced by CLA and related compounds (M. Degli Esposti, unpublished data). See the work of Yu and co-workers [7] for a method using MCLA to measure the superoxide generation of isolated respiratory complexes.

The second procedure is based on the change in fluorescence of a probe normally used for DNA staining, ethidium bromide. Its reduced form, often labeled dihydroethidine (DHE) or hydroethidine, is specifically oxidized by superoxide anions [8] and has become one of the most frequently used probes for measuring cellular superoxide [9–12]. With isolated mitochondria, the problems derived from the binding of DHE to DNA, which may seriously complicate the data obtained on living cells [11], are minimal. Valuable protocols for superoxide measurements with DHE are described in the recent reports of Nicholls and co-workers [11,12].

2.2. Hydrogen peroxide

The first method that was introduced to measure H_2O_2 production by isolated mitochondria is based on the quenching of the fluorophore scopoletin resulting from its reaction with hydrogen peroxide and peroxidases [13]. This method is still relatively popular (e.g., [14]), but suffers from two major problems: a relatively low sensitivity, and strong intereference by endogenous NAD(P)H fluorescence. In addition, scopoletin fluorescence cannot be exploited for ROS measurements in living cells [5].

A fluorescence-based method that was introduced very early in the literature [15,16] has been reapplied to ROS measurements in mitochondria [5]. It is based on the fluorogenic probe dichlorodihydrofluorescein diacetate (DCFDA or H_2DCF). The principle for the DCFDA method is opposite that of the scopoletin method, since ROS detection is proportional to an increase in the basal level of the probe, which emits an intense green fluorescence only after deacylation and subsequent oxidation, predominantly by H_2O_2 [15–18]. The fluorescent product, DCF, can be accurately quantitated by standard calibrations and, contrary to other fluorescent probes [19], does not inhibit the activity of complex I at the concentrations suitable for ROS assays. Fig. 1 shows a direct comparison of the scopoletin and DCFDA method, illustrating the superior sensitivity of hydrogen peroxide detection using the fluorogenic DCFDA. A major advantage of this method is that it is equally applicable to mitochondrial preparations and living cells [5,20,21], and a standardized protocol is provided here.

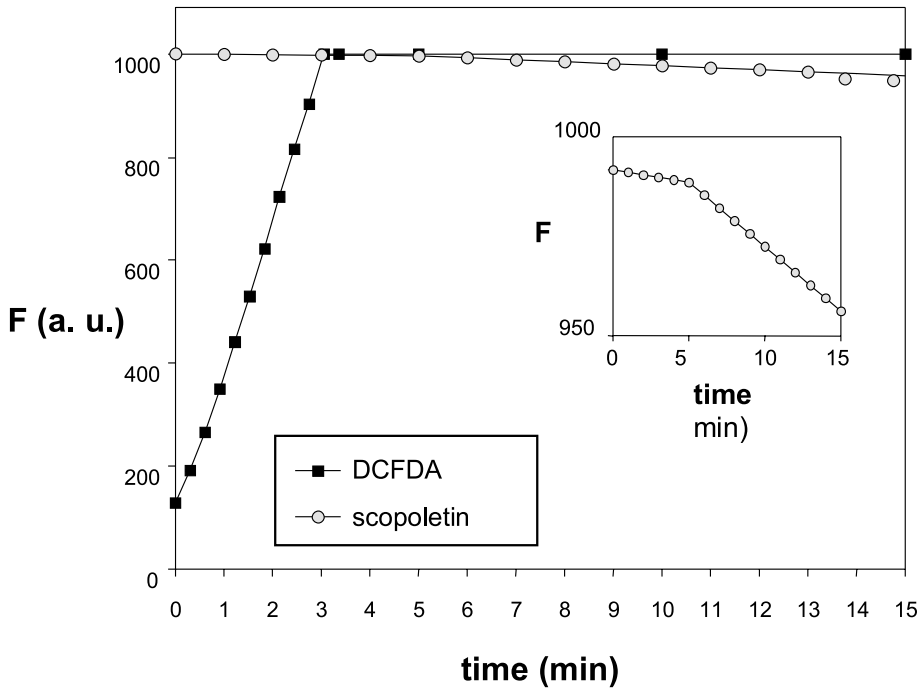

Fig. 1. Measurements of ROS with DCFDA were compared directly with those obtained with scopoletin using HRP and the direct addition of hydrogen peroxide at a saturating concentration (200 mM) [26]. Both probes were at a final concentration of 1 μM in the medium (standard phosphate–EDTA buffer; cf. [20,21]). Note the low rate of scopoletin compared with that of DCFDA under the same experimental conditions (scopoletin data are magnified in the inset). Similar results were obtained in the presence of mitochondrial preparations and without HRP [26].

2.3. Protocol for DCFDA assay

ROS production by mitochondrial preparations is measured in either spectrofluorometers or fluorescence plate readers [20–22]. With plate readers it is possible to obtain a large number of data with multiple replicates under the same conditions, thereby allowing the analysis of several variables and conditions. In a typical assay, each well of a 96-well microtiter plate (made of material optically suitable for fluorescence measurements) is filled with phosphate or phosphate-buffered saline (PBS) buffer, containing 1 μM of DCFDA (diluted from a stock solution in dimethyl sulfoxide (DMSO)) and 0.5 mg/ml of submitochondrial particles or mitochondria, to a final volume of 0.15–0.2 ml. The reaction is usually started by the addition mitochondrial substrates, e.g., 10 mM succinate, and followed, after mixing the reagents using application for automated shaking, for at least 30 min at the desired temperature (e.g., 30 °C). The fluorescence readings can be set at various wavelengths in the region 500–540 nm, depending on the technical specifications of fluorometers or plate readers. In recent work, measurements were routinely obtained with excitation wavelength at 485 nm and emission wavelength at 520 nm, with 5-nm bandwiths [20–22]. Samples can be further supplemented with exogenous peroxidases, e.g., 1 unit/ml horseradish peroxidase (HRP, Sigma) to enhance severalfold the rates of DCFDA oxidation by mitochondrial preparations, without changing the qualitative effect of respiratory inhibitors. Inhibitors, when used, are generally added in the reaction medium of the well before addition of the biological preparation, to minimize DCFDA oxidation by the radicals produced with endogenous substrates. Quantitative evaluation of the DCF fluorescence measured by the plate reader must be undertaken with a serial dilution under the same conditions of the experiments using commercial DCF [15,20]. These calibrations can remain linear in ranges spanning subnanomolar to submicromolar concentrations of DCFDA, depending on the electronic and optical capacity of the instrument available.

Since HRP and other peroxidases also increase the spontaneous oxidation of DCFDA without mitochondria, and the method is extremely sensitive, measurements can be easily obtained in the absence of external peroxidases. In general, DCFDA measurements are more than 80% sensitive to hydrogen peroxide and partially sensitive also to the hydroxyl radical [21]. The contribution of other ROS, including organic hydroperoxides, to DCFDA oxidation in the presence of mitochondria is generally minimal.

3. ROS measurements in live cells

Because mitochondria are the predominant generators of ROS in animal cells, measurement of cellular ROS chiefly reflects mitochondrial function, or dysfunction. The major radicals that can be measured within cells are superoxide, hydrogen peroxide, and lipid (hydro)peroxides. In general, the quantitative analysis of these radicals within cells is difficult due to the high intracellular concentration of glutathione, which can form thiyl or sulfinyl radicals, the variable concentration of metals, which can either catalyze or inhibit radical reactions, and the presence of free radical–quenching agents such as spermine and albumins. Taking into account these potential difficulties, several approaches can be used to evaluate ROS production by living cells [5]. Appropriate methods can provide a valuable quantitative analysis, especially by comparing control and stress-treated cells with accurate normalization of the results.

Superoxide is the first radical species produced by mitochondria, but its detection in live cells can be heavily affected by the expression level of Mn-SOD (superoxide dismutase), which is a highly regulated antioxidant scavenger present in mitochondria. The expression level of Mn-SOD varies greatly among different cell lines and also within a given cell line, depending on metabolic or stress (e.g., death receptor-induced apoptosis) conditions. Dihydroethidine is the most sensitive and most frequently used probe for measuring cellular superoxide [9–12], even if it suffers from complications derived from binding to mitochondrial and nuclear DNA, and indirect intereferences from changes in mitochondrial membrane potential [11]. These problems can be reduced by using concentrations of the probe around 1 μM in the assay medium [11].

Researchers generally prefer measurements of hydrogen peroxide when studying mitochondrial ROS in live cells. One good reason for this preference is that there is an increasing variety of probes that can detect cellular hydrogen peroxide (see the relevant chapter in the current online version of Molecular Probes, http://www.probes.com/handbook/sections/1900). Moreover, hydrogen peroxide is constantly produced and, owing to its high stability, maintained at detectable steady-state levels within metabolically active cells. A problem that one certainly faces is to choose probes and methods that can be most appropriate for the particular application of interest. Several of the ROS-sensitive probes have been developed for measuring nonmitochondrial reactions, especially the oxygen burst of macrophages, and are not necessarily appropriate for measuring mitochondrial hydrogen peroxide. Moreover, many probes induce inhibition of complex I at the concentrations suitable for ROS detection in cells [5,19]. This inhibition should be avoided, because it leads to altered or enhanced ROS production by mitochondria [21,22].

The most widely used probe for measuring cellular production of hydrogen peroxide is DCFDA [5]. Despite criticisms related to cell leakage [23] and

potential interference by cytochrome *c* and other iron proteins [24,25], DCDFA measurements can provide robust and reliable measurements of mitochondrial production of hydrogen peroxide in (nonmacrophage) cells [17,18,20,22]. This is testified by recent quantitative data obtained in different cell systems and under different measurement conditions (e.g., [26–28]). The standard protocol for DCDFA measurements with either adherent cells [20] or cells in suspension [22] is described below in the microtiter plate format. Each 96-well plate is supplemented with $0.5–5 \times 10^5$/ml cells in PBS (or another appropriate medium), and incubated with 1–2 μM DCDFA (diluted in the medium from a 5 mM stock solution in DMSO) for 10–15 min in the dark. Measurements are usually initiated by the addition of nutrients like glutamine and glucose, and can follow appropriate manipulations of mitochondrial or cell function (cf. [20,28]). Using excitation at 485–495 nm and emission at 520–530 nm (depending on the instrument specifications and available filters), fluorescence is then recorded every 1 or 2 min for at least 30 min, taking care that excitation light does not perturb cell viability (this can be checked at the end of the reaction by trypan blue staining or by counting the floating cells when adherent cells are used). Blanks containing equivalent concentrations of dead cells (prepared, most conveniently, by freezing and thawing an aliquot of the cell suspension [20]) are required to evaluate the background oxidation of DCFDA, which is generally low, especially if either serum or albumin is present in the assay medium [26]. Interestingly, dead cells are unable to efficiently oxidize DCFDA, for reasons that are not fully understood.

Given the various problems that can complicate an accurate evaluation of DCFDA data on live cells [21–26], integration of this method with another procedure using a different probe that is most specific for mitochondrial radicals is recommended. Molecular Probes has recently developed a number of mitochondria-specific dyes that can be used to this end, and I present here a well-tested protocol for ROS-specific staining of mitochondria using reduced rosamine probes [5,22,29]. Reduced Mito Tracker Red (CM-H_2XRos) produces a ROS-specific staining of mitochondria within cells that is amenable to both qualitative and quantitative measurements of mitochondrial production of hydrogen peroxide, and possibly also lipid hydroperoxides. CM-H_2XRos does not fluoresce until it enters an actively respiring cell, where it is oxidized predominantly by reactions involving hydrogen peroxide production. Owing to its positive charge acquired on oxidation by intracellular ROS, the probe covalently binds to mitochondrial proteins and thus produces a permanent organelle-specific stain. Of note, if mitochondrial membrane potential collapses during cell incubation and permeation of the probe, red staining can diffuse to other cellular compartments, especially the nucleus. To achieve effective ROS-sensitive staining, cells are suspended at $0.4–1 \times 10^6$/ml in fresh growth medium containing 0.5 μM CM-H_2XRos, which has been prepared just before the experiment by dissolving the content of a commercial vial in 0.1 ml of pure DMSO. Cell are incubated in this staining medium for 15 min at room temperature, washed twice with PBS, and then fixed with conventional procedures (e.g., with a fresh solution of 3.7% formaldehyde in PBS [5,22]).

After being washed and mounted on slides, the stained cells are analyzed by cytofluorescence techniques. Confocal microscopy should be undertaken with intermediate photomultiplier voltage and a 590-nm-bandpass filter for fluorescence emission [5]. The basal fluorescence intensity may vary significantly in different types of cells and under different conditions of stimulating ROS production. For epifluorescence analysis, long-wavelength emission should be used, for instance, the Texas Red filter, to effectively evaluate the fluorescence of the rosamine probe [5,29].

4. New perspectives in the measurement of lipid peroxidation

Mitochondrial ROS production inevitably produces lipid and other organic hydroperoxides that may accumulate with aging, during cell signaling, or under pathological conditions [1]. Lipid peroxidation is the ultimate noxious product of oxygen radicals that impairs mitochondrial and cellular function, fundamentally by damaging membranes [30]. Despite the importance of obtaining detailed estimates of lipid peroxidation in mitochondria and cells, the methods most frequently used are poorly sensitive or not sufficiently validated. These methods are based on the detection of secondary reaction products, mainly 4-hydroxy-2-nonenal (malondialdehyde) [30–32]. Clearly, there is ample scope to introduce and divulgate effective and sensitive methods for measuring lipid peroxidation, and here I briefly review some new directions.

cis-Parinaric acid is a natural fluorescent lipid that has been applied to evaluate lipid oxidation in cells [33,34]. With regard to mitochondrial lipid oxidation, only a few studies have been published [34,35], presumably due to the difficulty of handling the *cis*-parinaric probe, which is extremely prone to spontaneous autoxidation. This and other difficulties have so far prevented the use of *cis*-parinaric acid in routine and robust assays for measuring mitochondrial lipid oxidation under normal aerobic conditions.

Hopes for improved and more sensitive methods for the measurement of cellular lipid oxidation arise from recent applications of BODIPY(2-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-pentanoyl)-

based lipid compounds. A novel assay for peroxyl radicals uses lipid derivatives conjugated to BODIPY dyes, such as BODIPY 581/591 hexadecanoic acid, to measure the antioxidant activity in liposomal systems [36]. This assay is based on the loss or shift of the probe fluorescence produced by its interaction with peroxyl radicals and the retention of the fluorescent signal in the presence of chain-terminating antioxidants that quench free radicals. Notably, variations of this assay could be suitable to measure lipid peroxidation in cells [37,38]. For instance, peroxyl radicals have been detected in cell membranes using BODIPY FL hexadecanoic acid, which exhibits the red shift common to the fluorescence of other BODIPY probes when concentrated in hydrophobic environments [37–39], thus permitting accurate measurements of lipid peroxidation in live cells. In principle, similar methods could be applied to measure the level of lipid peroxidation in mitochondrial membranes, either in vitro or within live cells, but no detailed study is yet available.

5. Interpretation of mitochondrial ROS production in relation to the contribution of redox groups

Irrespective of the method use to evaluate mitochondrial ROS, the interpretation of the results requires critical attention. It is generally accepted that radicals derived from the partial reduction of ubiquinone (Q) are predominant generators of ROS during mitochondrial respiration [1–3,14,21,40,41]. The various dehydrogenases that reduce ubiquinone produce protein-stabilized semiquinone (SQ) intermediates as part of their catalytic cycle. Every hundred turnovers or so, one of these intermediates can spontaneously react with oxygen instead of being fully reduced or oxidized by the redox groups of the dehydrogenases. This autoxidation leads to the production of superoxide and other radicals, including lipid hydroperoxides; ubiquinone is dispersed within the lipid membrane and thus the phospholipids in contact with the SQ radicals may become primary targets of ROS-induced peroxidation. High levels of SQ intermediates are maintained when mitochondria respire under conditions of low energy demand (e.g., state IV respiration), which slow down the electron flow due to the thermodynamic back-pressure exerted by high membrane potential [14,40,41]. This is why partial (e.g., by exogenous ADP or calcium) or permanent (by uncouplers) collapse of membrane potential strongly decreases the mitochondrial production of ROS [13,14,40]. However, a basal level of ROS production is present also in fully uncoupled preparations of mitochondria, as clearly demonstrated with the DCFDA method [20,21,26] and other procedures [14,42].

A dissection of the relative contribution of individual respiratory components to the mitochondrial (or cellular) production of ROS can be obtained using specific respiratory inhibitors, but care must be taken in interpreting the results. For instance, a classic inhibitor such as rotenone, if used at concentrations far exceeding its specific site in complex I, produces spurious ROS that do not derive from the inhibited enzyme complex [21,26,32]. Nevertheless complex I, as well as complex II [21], is a major generator of ROS and lipid peroxides in mitochondria and living cells [1,2,20–22,32,35]. The relative content of these complexes, which is different in different tissues, and the variations in the metabolic dominance of respiratory substrates (e.g., glutamine in white blood cells, but fatty acids in renal cells) determine the contribution of each dehydrogenase to the overall production of mitochondrial ROS in the cell system under study.

Of note, the role of complex II in mitochondrial ROS has been often neglected owing to the effects of antimycin, a powerful complex III inhibitor that invariably enhances ROS production [13,14,20,21,40–42]. The effect of antimycin has been interpreted to arise from the autoxidation of the SQ intermediate formed by complex III at a site distinct from that of the inhibitor, namely, center **o** [1,14,40,41]. However, this SQ is extremely unstable in either the presence or the absence of the inhibitor [43,44] and, consequently, unlikely to contribute extensively to the antimycin-induced production of ROS [21]. In contrast, the SQ radicals produced by complex II are stabilized by downstream inhibition with antimycin [43,45], and are thus likely to be responsible for the enhanced ROS production detected when complex III is inhibited [7,21,44–47]. Moreover, the contribution of complex III to the basal production of mitochondrial ROS is relatively small in comparison to that of complex II [21,42], especially in cultivated cells [21,26].

To conclude, the reactions and respiratory components that reduce Q should be considered the predominant generators of ROS, as well as of lipid peroxidation, in mitochondria, unless other cellular defects are evident.

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