Review

Determination of mitochondrial reactive oxygen species: methodological aspects

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Abstract

The generation of Reactive Oxygen Species (ROS) as by-products in mitochondria Electron Transport Chain (ETC) has long been admitted as the cost of aerobic energy metabolism with oxidative damages as consequence. The purpose of this methodological review is to present some of the most widespread methods of ROS generation and to underline the limitations as well as some problems, identified with some experiments as examples, in the interpretation of such results. There is now no doubt that besides their pejorative role, ROS are involved in a variety of cellular processes for the continuous adaptation of the cell to its environment. Because ROS metabolism is a complex area (low production, instability of species, efficient antioxidant defense system, several places of production...) bias, variances and limitations in ROS measurements must be recognized in order to avoid artefactual conclusions, and especially to improve our understanding of physiological and pathophysiological mechanisms of such phenomenon.

Keywords: ROS • mitochondria • oxidative stress • redox status • fluorescence

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Introduction

For the chemist, Reactive Oxygen Species (ROS) result from dioxygen chemical characteristics and for the physiologist such pathway is viewed as an inevitable consequence of aerobic metabolism [1, 2]. Indeed, the generation of such species has long been admitted as the cost of aerobic energy metabolism, with oxidative damage as unavoidable consequence. Under physiological conditions, the deleterious effects are minimized by antioxidant defenses either by preventing their formation, by scavenging the reactive species or by repairing the damage caused to the biological molecules [3]. Mitochondrial oxydative phosphorylation pathway is the major site of ROS production as by-products of the electron transport chain. Mitochondrion metabolism permits to adequately match ATP synthesis to cellular energy demand according to physiological and/or pathological constraints. This adaptation occurs not only in terms of changing the activity of oxidative phosphorylation pathway, but also in terms of efficiency of this pathway, mitochondrial ATP production being a permanent compromise between rate and efficiency [4].

Yet there is increasing evidence that ROS plays an essential function in the continuous adaptation of the cell to its environment. ROS are involved in a variety of cellular processes from cell proliferation to cell adaptation to hypoxia [5], from apoptosis to carcinogenesis, to maintain or reestablish redox homeostasis, acting as intracellular second messengers or modulating signal transduction pathways [6]. Indeed, if oxygen consumption and ATP synthesis are modulated by energy demand, cells seem to be also able to adapt O_2 consumption to available O_2 (*i.e.* to oxygen tension in the surroundings). Mitochondria may function as an O_2 sensor by modulating the generation of ROS [5, 7, 8].

Oxidative stress resulting from redox state “dysregulation” has been implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, ischemia/reperfusion injury and many other diseases. Therefore, redox regulation might occupy an increasing place in the future of clinical practice. Even healthy people are exposed to a continuous oxidative stress, and the “the free radical theory of

aging” is probably one of the most documented concerning the phenomenon of aging [9]. Hence, the use of reliable methods to assess ROS production becomes mandatory for the biological research in this area. Available methods are numerous, they are based on different technological approaches and have different applications according to the experimental objectives. The purpose of this methodological review is to present some of the most widespread methods of ROS generation measurements and to underline the limitations as well as some problems in the interpretation of such results.

How, when and why are ROS produced in mitochondria?

Electron transport chain (ETC) of mitochondria is a permanent source of reactive oxygen species (ROS) in cells. About 1 to 3 % of total mitochondrial oxygen consumed is incompletely reduced and leads to ROS production [1, 2]. By the transfer of one single free electron to molecular oxygen at the level of NADH CoQ reductase (Complex I) and CoQ cytochrome C reductase (Complex III), monoelectronic reduction of O_2 generates ($O_2^{\bullet-}$) [10]. This superoxide anion is scavenged by intramitochondrial Super Oxide Dismutase (Mn SOD) leading to hydrogen peroxyde (H_2O_2) and in the presence of reduced transition metals (such as iron) to the highly reactive hydroxyl radical ($OH^{\bullet}$). It is generally accepted that following its generation in the inner mitochondrial membrane, at the level of the respiratory chain, superoxide ions are vectorially released into the mitochondrial matrix and subsequently dismutated in hydrogen peroxide by the high-level matrix enzyme Mn SOD. Conversely to the low diffusion capacity of the charged superoxide anion, hydrogen peroxide can easily cross the mitochondrial membrane towards cytoplasm [11, 12]. It should be added that a direct release of superoxide anion into the intermembrane space has been recently proposed [13]. Similarly to the mitochondrial super oxide ion pathway, in the cytosol the scavenging of $O_2^{\bullet-}$ to H_2O_2 is permitted by an equivalent enzyme: CuZn SOD [14].

Fig. 1 ROS production and ROS protective system in mitochondria.

These data are shown in Fig. 1.

The deleterious effects of ROS are counteracted by several mechanisms permitting an efficient antioxidant defense. Under physiological conditions antioxidant capacity neutralizes ROS production. But when an excess of ROS is generated (ETC dysfunction, ischemia/reperfusion, apoptotic process...) or if the antioxidant defense system is overflowed (malnutrition, genetic disease, neurodegenerative disease, ...), an oxidative stress is created corresponding to the detrimental effect of ROS. Mitochondria represent the first target [15] (Fig. 2). Alterations of mitochondrial membrane are the consequence of lipid peroxidation and thiol residue damage. Mitochondrial DNA is particularly sensitive to ROS because of the lack of histone protective system as is the case of nuclear DNA. These alterations result in mitochondrial dysfunction with (i) decreased ATP production (ROS injury on ETC) [16], (ii) membrane

permeabilization (ROS injury on lipid membrane) [17], (iii) mtDNA rearrangements [18] leading to various mitochondrial diseases [19].

Apart from a role as destructive agents, ROS act also as cellular messengers and accumulating evidences suggest that ROS are essential in cell signaling and regulation. Current concepts of ROS signaling can be divided in two general mechanisms of action: alterations in intracellular redox state [20] and oxidative modification of proteins (for recent review see [21]). ROS are probably necessary as regulation mediators for cell growth control and there is evidence that protein oxidation plays a significant role in cell differentiation (for review see [22]). The mechanisms relating ROS and the regulation of gene expression have been recently reviewed [23] and several studies focus the role of ROS in the induction of mitochondrial permeability transition [24, 25], apoptosis [26-28] or in aging [9, 29, 30].

Fig. 2 ROS as inducers in oxidative stress, apoptosis initiation and cell signalling.

Methods for ROS measurement

The three reactive oxygen species: $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$, are linked by spontaneous or enzymatic dismutation, therefore the question arises as to which species must be measured. Among the different reactive oxygen species, $OH^{\bullet}$ is probably the most unstable with a half-life of 10^{-9} sec [31]. This compound is so reactive that it reacts with any reactive species in the neighborhood of approximately 30 Å. H_2O_2 is more stable, but it is highly metabolized either enzymatically by catalase and GSH peroxidase, or non enzymatically by low concentrations of transitional metals such as iron. Thus, the lifetime of H_2O_2 in the cell is depending on the concentration of protective enzymes in the close environment. Meanwhile, due to its high diffusibility, H_2O_2 is able to cross the membranes (and may diffuse).

The lifetime of $O_2^{\bullet-}$ cannot be calculated without a precise knowledge of local concentration of SOD and other substrates. However, the lifetime of $O_2^{\bullet-}$ in a cellular environment is expected to be very short. ROS can be stabilized by reducing the scavenging system (Table 1). But in this case, other metabolites can be formed from cyanide (CN^-) or azide (N_3^-), such as cyanyl ($CN^{\bullet}$) or azidyl ($N_3^{\bullet}$) radicals respectively, which can interfere with ROS measurement [32]. Moreover, cyanide and azide are well know inhibitors of cytochrome C oxidase (ETC complex IV) and of horseradish peroxidase, often used in ROS measurement methods [33].

A number of direct or indirect analytical methods have been proposed for the detection and/or the quantification of ROS generation and only the most common methodologies used to assess the mitochondrial generation of ROS in cells are reviewed in this paper.

Table 1 Antioxidant protection system, sub-cellular localisation and main inhibitors.

	CELLULAR LOCALISATION	INHIBITOR
SOD (EC.1.15.1.1)		
SOD-Mn	Mitochondria (Matrix) Peroxisomes	Metal Chelator (Mn, Cu, Zn) Cyanide
SOD-CuZn	Cytoplasm Mitochondria (Intermembrane Space) Peroxisomes (?)	Azide Diethyldithiocarbamate (Cu, Zn)
CATALASE (EC.1.11.1.6)	Mitochondria matrix (rat heart) Cytoplasm Peroxisomes	3-Amino 1,2,4-triazole - ATZ Azide Cyanide
GLUTATHIONE PEROXIDASE (EC.1.11.1.9)	Mitochondria Cytoplasm Nucleus Peroxisomes	Mercapto succinic acid Penicillamine Adenosine
FENTON REACTION	Cytoplasm Mitochondria	Polyvalent anions Diethylenetriaminepentaacetic DTPA Deferoxamine - DF Ethylenediaminetetacetic - EDTA o-Phenanthroline (Fe2+)

All informations about enzymes are extracted from the Web database "BRENDA : the comprehensive enzyme information system" <http://brenda.bc.uni-koeln.de/>

Reactive oxygen species exist mainly as low to very low concentrations (from 10^{-4} to 10^{-9} M). This implies the use of highly sensitive analytical techniques.

Spectrophotometric methods

For the detection of ROS, spectrophotometric methods have first been used. They are based on the alteration of absorption characteristics of a chromogen after interaction with ROS. Widespread examples are the reduction of tetrazolium salts for formazan [34], the reduction of ferricytochrome C [35]. Other chromogens used are epinephrine [36, 37] or adrenaline [38] for $O_2^{\bullet-}$. These methods are still useful in some specific experimental situations. As an example, biological membranes are

impermeable to ferricytochrome C because of its size. Therefore, its use represents an adequate measurement of extra cellular $O_2^{\bullet-}$ production. But since $O_2^{\bullet-}$ only poorly diffuses through biological membranes, ferricytochrome C reduction requires the use of submitochondrial particles or isolated complexes [39]. Nevertheless, in the complex biological systems the actual ROS production is buffered by the protective system and the "net" ROS production, which is the parameter of interest, is very low. Hence, in such situations more sensitive methods are required.

Fluorimetric methods

Detection of H_2O_2 , $O_2^{\bullet-}$ or $OH^{\bullet}$ is also possible by fluorescent methods. The common principle is

Fig. 3 Interfering products with DCF-DA measurements. Dichlorofluorescein (DCF) fluorescence measurement [excitation and emission measurement: 500 and 521 nm respectively; concentration of dichlorofluorescein diacetate (DCFH-DA): 5 μ M] was achieved with a PTI Quantamaster C61 at 30°C with continuous stirring, the incubation medium was 250 mM sucrose, 10 mM tris-HCl (ST medium, pH 7.4). These measurements were achieved in absence of mitochondrial material. The ST medium was supplemented after 5 min with DCFH-DA 5 μ M and esterase 10 UI. Tested products were added in indicated concentrations and the results were calculated as fluorescence enhancing by unit of time relative to the fluorescence enhancing by unit of time of the control (without added product) measured in the same conditions. Substrates series A, 1: Dihydroxyacetone (DHA) 20 mM, 2: Proline 20 mM, 3: Lactate/Pyruvate 20 mM/2 mM, 4: Glucose 20 mM, 5: Glutamate/Malate 5 mM/2.5 mM, 6: Succinate/Malate 5 mM/0.5 mM, 7: Octanoate 2 mM, 8: NADH 2.5 mM, 9: ADP/Pi 1 mM/5 mM, 10: ADP 1 mM, 11: Pi 5 mM. Inhibitors series B, 12: Myxothiazol 4 μ M, 13: Antimycine A 1 μ M, 14: Rotenone 2.5 μ M. Others, 15: Digitonine 60 μ M, 16: BSA 1%.

based on the properties of probes, fluorescence of which varies according to their redox state. One of these fluorescent methods is based on the ability of heme peroxidase to catalyze H_2O_2 dependent dimerization of substituted phenolic compounds. The main advantage of this method, when using horseradish peroxidase, is the high specificity and the sensitivity regarding H_2O_2 determination. With scopoletin, the fluorescent compound is oxidized in the presence of horseradish peroxidase by H_2O_2 to a non-fluorescent reduced derivative. Although this is a specific method, it is based on fluorescence decrease, *i.e.* a non very accurate determination [40]. Increasing fluorescence with this method requires the use of p-hydroxyphenylacetate (PHPA) [41] or homovanillic acid [42] as substrates. Numerous published applications demonstrate the interest of such methods [29]. A

recent improvement of the method permits to avoid interference of the detection system with any mitochondrial constituents [43, 44].

A similar procedure permits to assess the unstable and poorly diffusible $O_2^{\bullet-}$ after dismutation by SOD leading to H_2O_2 . Hydroethidine (HET), a specific indicator of $O_2^{\bullet-}$, has been shown to be specifically oxidized to fluorescent ethidium [45].

Fluorescent methods to detect hydroxyl radicals are difficult and an indirect method requires the separation of fluorescent radicals by high-performance liquid chromatography [46].

One of the widespread methods to detect H_2O_2 production by mitochondria is based on 2',7'-dichlorofluorescein (DCF- H_2) oxidation. This probe has low basal fluorescence but, when oxidized, it is converted into 2',7'-dichlorofluorescein (DCF), a high fluorescent component

Fig. 4 Signal specificity measurements. Rat liver mitochondria (RLM) were prepared according to standard differential centrifugation procedure in a medium containing 250 mM sucrose, 20 mM tris-HCl (pH 7.4) and 1 mM EGTA (STE medium). Uncoupled submitochondrial particles (SMP) were formed after sonication in an ice bath three times for 15 sec of frozen/thawed RLM in STE medium and were isolated with two centrifugations at 100000 g for 45 min. The pellet was re-suspended in STE medium and stored at $-80^{\circ}C$ until use (Figure 2 and Figure 3). These measurements were achieved in the presence of 125 $\mu g/ml$ submitochondrial particles in the ST medium supplemented with DCFH-DA 5 μM , Pi 2.5 mM, EGTA 1 mM, $MgCl_2$ 1 mM. **A:** after 1 min 10 UI esterase were added; after 5 min 1mM aminotriazole (ATZ, catalase inhibitor) was added; after 10 min 2.5 mM succinate (top) vs the same volume of ST medium (under) was added. **B:** after 5 min 1mM aminotriazole (ATZ, catalase inhibitor) was added; after 10 min 2.5 mM succinate (top) vs the same volume of ST medium (under) was added. **C:** after 5 min 1mM aminotriazole (ATZ, catalase inhibitor) and 0.5 μM antimycin A were added; after 10 min 2.5 mM succinate (top) vs the same volume of ST medium (under) was added.

Fig. 5 Signal sensitivity measurements. These measurements were achieved in the presence of 62.5 $\mu\text{g/ml}$ (A) or 125 $\mu\text{g/ml}$ (B) of submitochondrial particles in the ST medium supplemented with DCFH-DA 5 μM . After 5 min 1mM aminotriazole (ATZ, catalase inhibitor) and 0.5 μM antimycin A were added in all samples; after 10 min 2.5 mM succinate (top) - (A) and (B) - vs the same volume of ST medium (under) - (A) and (B) - was added.

(λ_{ex} 500nm – λ_{em} 520nm). The dichlorofluorescein-di-acetate (*i.e.* the acetylated reduced form) derivative is permeable and diffuses into cells, but once internalized, it is cleaved by intracellular esterases in dichlorofluorescein [47]. The main interest of this method is its simplicity, but it is limited by a low specificity: it may be an indicator of cellular redox status rather than a real estimation of intracellular H_2O_2 production. Moreover, some technical problems have been reported, resulting from enzymatic or non-enzymatic pathways (H_2O_2) [48, 49]. The specificity of this method is questionable since peroxynitrite is also detected with a high sensitivity [50]. Moreover, other oxidized products such as trolox

[51] or other low molecular weight oxidants [52] are also detected. Finally, the observation of DCF fluorescence, even if H_2O_2 is omitted from the medium represents a very negative finding. Indeed, it has been demonstrated that the photoreduction of DCF results in the formation of DCF semiquinone free radical ($\text{DCF}^\bullet$) while DCFH can be oxidized to $\text{DCF}^\bullet$. Under aerobic conditions, the free radical ($\text{DCF}^\bullet$) is oxidized to DCF generating superoxide radicals [53]. Hence, DCF permits a *de novo* ROS formation independantly from reducing agents in the milieu, leading to the conclusion that “DCFH cannot be used conclusively to measure H_2O_2 formation in cells” [53]. A more recent work has stressed the

effect of cytochrome C as potent catalyst of DCF formation, suggesting that the fluorescence observed in dying cells could be related to increased cytosolic cytochrome C content [54]. Despite these considerations, limiting the validity of the method, several applications have been proposed and the determination of fluorescence is performed commonly with a fluorimeter [55-58], either a flow cytofluorimeter [59], an image analysis system [60], or a confocal microscope [61].

EPR spectroscopy has the specific advantage of a direct method for free radical detection (for a recent review see [62]). The unpaired electron confers to free radicals the property of paramagnetic resonance. The low sensitivity of measurement is largely compensated by the addition of a *spin-trap* leading to a stable radical compound, which accumulates until detectable range. Nitrones and nitroso compounds are classical *spin-traps*, producing, in the presence of $O_2^{\bullet-}$ or $OH^{\bullet}$ specific nitroxyl radicals. The main limitation of this method is the availability of a specific and expensive equipment, which is not usually present in biological laboratories.

$O_2^{\bullet-}$ measurement can be achieved with the use of chemiluminescence resulting from energy transfer between molecules during redox reactions. Luminol and lucigenin have been used extensively as chemiluminescent substrates in various cellular systems. After reduction of these compounds, a reaction with $O_2^{\bullet-}$ leads to an unstable intermediate which emits light during the spontaneous decomposition to its ground-state electronic configuration [63]. Such method is extremely sensitive and has been used to measure $O_2^{\bullet-}$ in intact cells, isolated mitochondria and submitochondrial particles [64-67].

Bias, variances and interferences in ROS measurements

The increasing interest of ROS related to their role in physiology and pathology is depending on the quality of methodologies used to detect and to quantify such unstable species. It must be admitted that the methods used in the literature to

assess reactive oxygen species in biological systems are multiple and, among the large number of published applications, many divergent results have been observed. This might not only be due to technical difficulties but also to a wide variety of experimental conditions such as: animal species, sex, age, physiological state, organ, type of biological material etc., which are not often considered.

A few studies only have emphasized the diversity and sometimes the variance of ROS measurements in animal species [68-70]. Moreover, production of ROS depends on the organ considered: brain, heart, muscle, kidney, liver, etc. [39, 71]. Location of ROS production in most studied organs (heart, brain, muscle) has been identified at complex I and complex III level, according to added substrates (glutamate – malate for complex I and succinate for complex II) and/or inhibitors used. The relative magnitude of ROS signal varies between tissues. When ROS are generated in complex I in the presence of rotenone, the largest signal occurs in heart [1]. But, when generated at the level of complex III, *i.e.* in the presence of complex II substrate succinate and with Antimycin A as complex III inhibitor, the largest ROS signal occurs in brain [72]. Recent studies in liver propose complex II as another site of ROS generation [57, 73, 74]. Only rat heart mitochondria contains catalase [75], while in all other species and organs this enzyme is located in cytosol and peroxisomes. These considerations are of importance when ROS production is regarded as integrated metabolism in intact cells, resulting from pro-oxidant/antioxidant balance due to the presence of multiple interferences. Moreover, the type of biological material used as ROS source is of great importance: intact organ or cells, permeabilized cells, mitochondria or submitochondrial particles or isolated electron transport chain complex, etc. are very different, regarding their content in antioxidant compounds.

The specific endogenous mechanisms responsible for generating ROS production by mitochondria are still poorly understood. A large number of physiological parameters have been related to ROS production: age [29, 39, 76, 77], caloric restriction [73], oxygen tension [8, 78], exercise [79], seasonal variation [80].

In most of the physiological situations corresponding to the experimental conditions, ROS production is very low and is enhanced with specific complex inhibitors, which in turn can affect the detection, particularly in the case of fluorescence [81]. Such interference with fluorescence must be systematically estimated to exclude any misinterpretation of results (Fig. 3). Isolated mitochondria from various tissues are differently affected by the respiratory inhibitors rotenone (complex I) and antimycin A (complex III) in both sensitivity and responsiveness [71].

The various methods of ROS detection have been evaluated concerning their qualitative and quantitative performances. Concerning DCF-DA [53], that is one of most common probe used for ROS measurement, the sensitivity of the signal is rarely considered although the reactivity of the probe is very complex with fluorescence signals of non linear structures [82]. However, it is experimentally possible to assess the quantitative limitations of ROS measurement by DCF-DA. In the experiments shown in Fig. 4 and Fig. 5, the high signal measured in the absence of substrate (dotted lines) is actually not a signal of ROS production and the ratio specific/non specific signal is very weak and must be considered with caution. A relative determination with a reference sample in identical conditions is the best way to avoid problems of signal versus noise ratio.

In conclusion, if there is no doubt about the fact that ROS metabolism plays a crucial role in cell regulation, our understanding of physiological and pathophysiological events related to this pathway strongly depends on technical consideration. Indeed, regulation of ROS metabolism is certainly a very complex area, but methodological inaccuracy and technical pitfalls may add to the confusion. In addition, in physiological conditions ROS production is very low, and therefore some “enhancing tricks” are needed to make this production detectable. However, these experimental conditions may lead to artefactual conclusions. Ideally, investigation of ROS metabolism must consider not only the net production or the balance oxidant/anti-oxidant status but more precisely location, kinetics, targets in order to differentiate each reactive oxygen species in cell metabolism.

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