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Measurement of Mitochondrial ROS Production

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Abstract

The significance of reactive oxygen species (ROS) as aggravating or primary factors in numerous pathologies is widely recognized, with mitochondria being considered the major intracellular source of ROS. It is not yet possible to routinely measure mitochondrial ROS in animals or cultured cells with a reasonable degree of certainty. However, at the level of isolated mitochondria, one can easily monitor and quantify the rate of ROS production, identify major sites of ROS production, and compare the rates of ROS production in mitochondria isolated from normal and diseased tissue. In this chapter, we describe in detail the most recent and reliable method to measure mitochondrial ROS as the rate of H₂O₂ emission. This method may be employed with minimal modifications to measure H₂O₂ production by mitochondria isolated from various tissues and under a wide variety of experimental conditions.

Keywords

Reactive oxygen species; Mitochondria; Amplex; Oxidative stress

1. Introduction

The significance of reactive oxygen species (ROS) as aggravating or primary factors in numerous pathologies, ischemia, excitotoxicity, neurodegenerative diseases, and senescence is widely recognized and extensively reviewed elsewhere (1–11). More recent data strongly suggest that ROS, and specifically mitochondria-generated ROS, are involved in physiological signaling cascades regulating various cellular and organ functions (3,12–14), with H₂O₂ being a chief messenger molecule (15).

Mitochondria are believed to be the major intracellular source of ROS. Several decades of research have firmly established that ROS production is inherent to mitochondrial oxidative metabolism. This literature has been extensively reviewed by us and others elsewhere (8,12,16–23).

Whereas the primary ROS species produced in mitochondria is thought to be superoxide (17,24), by its chemical nature it is neither particularly reactive (21) nor capable of “long-distance” travel because it does not easily permeate cell and mitochondrial membranes due to its negative charge (17,21). However, its electrically neutral dismutation product, H₂O₂, is more reactive toward critical cellular targets such as protein SH groups and it is known to be a precursor of even more aggressive ROS, hydroxyl radical. Measuring H₂O₂ emission is a preferred and well-established method of evaluating mitochondrial ROS production (17,25–35). It is justified because (see for details (17)) most of the superoxide produced in mitochondria is almost immediately converted into H₂O₂ by the matrix-located superoxide dismutase (MnSOD) or dismutates spontaneously with a high rate into H₂O₂. Moreover, some mitochondrial sites such as flavins may produce H₂O₂ directly. Perhaps the most important reason to measure mitochondrial ROS as H₂O₂ emission is that methods to measure it are more quantitative and less labor-consuming and equipment-demanding than those to detect superoxide.

In this chapter, we describe in details the most recent and reliable method to measure low levels of H_2O_2 in vitro. It employs horseradish peroxidase to trap emitted H_2O_2 with high selectivity and affinity and Amplex Red Ultra (a derivative of 10-acetyl-3,7-dihydroxyphenoxazine) as a sensitive fluorescent probe for H_2O_2 (36).

2. Materials

1. Isolated mitochondria (see Note 1).
2. Incubation buffer: 125 mM KCl, 4 mM KH_2PO_4 , 14 mM NaCl, 20 mM HEPES-NaOH, pH 7.2, 1 mM MgCl_2 , 0.2% of fatty acids free bovine serum albumin, and 0.020 mM EGTA (see Note 2).
3. Amplex Red Ultra (Invitrogen) (see Notes 3, 5).
4. Horseradish peroxidase (e.g. Type VI-A, essentially salt-free, lyophilized powder, ~1,000 units/mg solid) (Sigma) (see Notes 4, 5).
5. H_2O_2 solution in water, 30–32 wt.%, semiconductor grade, 99.999% trace metals basis (Sigma).
6. Respiratory substrates and inhibitors (see Notes 5, 6).
7. (Optional) Catalase (e.g. from bovine liver, lyophilized powder, ~10,000 units/mg protein) (Sigma) (see Notes 5, 7).
8. (Optional) Sodium Azide (Sigma) (see Note 8).
9. (Optional) Cu, Zn Superoxide dismutase (e.g. from bovine erythrocytes, 2,500–7,000 units/mg protein, lyophilized powder) (Sigma) (see Note 9).
10. Small (5–10 ml) glass tubes.
11. Glass (preferable) or plastic disposable cuvettes with all walls transparent and flat bottom and matching stirring bars.
12. A quartz cuvette.
13. Fluorimeter equipped with a stirred thermostated cuvette holder such as “Hitachi F2500, F4700, or F8000 models (see Note 10).
14. Spectrophotometer capable of quantitative absorbance detection at 240 nm wavelength.
15. Common labware such as pipettors and tips.

3. Methods

3.1. Prepare the H_2O_2 Calibration Solution

Fill two glass tubes with 5 ml of de-ionized water, each. To the first tube, add 5 μl of 30% (v/v) H_2O_2 solution (1:1,000 dilution), mix well. Determine the concentration of H_2O_2 spectro-photometrically by placing 2 ml of this solution in a quartz cuvette and reading its absorbance at 240 nm; calculate the concentration of H_2O_2 employing the extinction coefficient $E_{240} = 43.6/\text{M}/\text{cm}$. Dilute H_2O_2 solution to ~0.1 mM by taking 50 μl of H_2O_2 solution from this tube and adding it to the second tube (1:100 dilution), mix well. Keep the second tube in ice and use within 1 h to calibrate the H_2O_2 assay (see Note 11).

3.2. Calibrate the Assay

Set the fluorimeter at 555 nm excitation and 581 nm emission wavelengths and turn on the cuvette holder's thermostat set at the desired temperature (25–37°C). Fill a cuvette with the incubation buffer (item 2, Subheading 2), add magnetic stirring bar, turn on the stirrer, and wait until the cuvette reaches the desired temperature (25–37°C). Add 4 U/ml of horseradish peroxidase, 10 μ M Amplex Red Ultra, 40 U/ml superoxide dismutase (optional, see Note 12) and mitochondria (0.03–0.1 mg/ml) to the cuvette. Record the fluorescence changes of Amplex for ~100 s. Make 6–8 additions of ~0.1 mM H₂O₂ by ~100 nmol (~1 μ l/ml) each, with about 30 s between the additions. You should obtain a “staircase”-like trace with about equal “steps” after each addition (see Fig. 1). Take the fluorescence numbers approximately at the points indicated by crosses (Fig. 1). Subtract the value of fluorescent signal right before the first addition of H₂O₂ from all other values and plot them vs. the amount of H₂O₂ added (Fig. 1). Calculate the slope coefficient of the graph. This coefficient is used to calculate the rate of H₂O₂ production by mitochondria, in nmols or pmols of H₂O₂ per mg mitochondrial protein per minute. The *R* square value should be at least 0.95.

3.3. Measure Mitochondrial H₂O₂ Emission

Fill a cuvette with the incubation buffer (item 2, Subheading 2, see Note 13), add magnetic stirring bar, turn on the stirrer, and wait until the cuvette reaches the desired temperature (25–37°C). Add 4 U/ml of horseradish peroxidase, 10 μ M Amplex Red Ultra, 40 U/ml superoxide dismutase (optional, see Note 12) and the same amount of mitochondria as used in step 3.3 to build the calibration curve. Record the fluorescence for ~150 s. Add respiratory substrates (see Note 6) and record H₂O₂ emission.

To illustrate a typical experimental protocol, Fig. 2 presents recordings of H₂O₂ production by isolated mouse brain mitochondria oxidizing NAD⁺-dependent substrates or succinate. The H₂O₂ generation is triggered by the addition of a respiratory substrate (succinate, Fig. 2a or pyruvate and malate, Fig. 2b, see Note 14). With NAD⁺-dependent substrates, H₂O₂ production was stimulated by rotenone, which inhibits NADH oxidation at Complex I (Fig. 2b). With succinate, rotenone inhibited H₂O₂ production indicating that it was fueled by reverse electron transfer from succinate to a site in Complex I (24). With either substrate, H₂O₂ production was stimulated by an inhibitor of Complex III (Antimycin A) (24,37).

3.4. Quantifying the Rates of H₂O₂ Emission

Calculate the rate of H₂O₂ emission using the slope coefficient obtained in step 3.3 by measuring the slope of the fluorescence traces in relative fluorescence units per minute and dividing it by the slope coefficient and by the amount of mitochondrial protein present in the cuvette. Present the rates of H₂O₂ emission in pmol/min/mg mitochondria.

3.5. Control Experiments (Optional)

If desired, two simple control experiments can be performed to ensure that what is measured is H₂O₂ and that the reaction is catalyzed by horseradish peroxidase (as opposed to some contaminants in your mitochondrial preparations). First, the incubation buffer can be supplemented with 200–400 U/ml of catalase. This should greatly suppress the observed rate of H₂O₂ emission, although not necessarily completely. Second, 1 mM azide (a strong inhibitor of horseradish peroxidase) can be included in the incubation medium; this should suppress the observed rate of H₂O₂ emission completely.

3.6. Interpreting the Obtained Data

Performing the assay as shown on Fig. 2 allows one to estimate the ROS production from several mitochondrial sites and can be interpreted in a reasonably straightforward way as

extensively discussed elsewhere (17,24–27,35,37,38). This protocol yields the rates of ROS production from Complex I of the respiratory chain in both forward and reversed electron flux conditions, the rate of ROS production by the Complex III, and that by the matrix dehydrogenases (see (17) for discussion). To note, ROS production capacity of mitochondria is controlled by the factors affecting and reflecting the metabolic state of intact mitochondria. It has been found that most important factors controlling the ROS production in mitochondria are the chemical nature of the respiratory substrates, the amplitude of the membrane potential, the pH of the mitochondrial matrix, and the oxygen tension in their surrounding (17,39). Perhaps the most important, irrespective of the nature of the respiratory substrate, is that the rates of ROS emission are directly related to the magnitude of the membrane potential of mitochondria (26,27,30,31,38); with NAD⁺-dependent substrates, the rate of ROS emission is modulated by the level of NAD(P)H reduction in mitochondria. Experimentally, this necessitates measuring the membrane potential of mitochondria in addition to their ROS emission rates when the latter are measured in the absence of respiratory chain inhibitors. However, further discussion of these issues is beyond the scope of this publication.

4. Notes

1. Quality of and purity of mitochondria is very important. Contamination of mitochondrial preparation with cytosolic structures such as peroxisomes, synaptic terminals, etc. and with fragments of broken mitochondria can significantly interfere with ROS measurements. In essence, a meaningful and reproducible ROS assay cannot be performed with crude mitochondrial pellets and requires at least some further purification of the mitochondrial preparation, for example, by an isopycnic density gradient.
 - a. A discussion of mitochondria isolation and purification procedures is beyond the scope of this manuscript. However, we feel compelled to briefly describe the most common procedure for isolation and purification of mouse brain mitochondria which was employed in experiments presented on Fig 2.
 - b. Non-synaptic mouse brain mitochondria were isolated by a modified isopycnic centrifugation procedure (40) employing Percoll density gradient. Briefly, cortex brain tissue was homogenized in the MSEGTA buffer comprising 225 mM mannitol, 75 mM sucrose, 20 mM HEPES, adjusted to pH 7.4, 1 mM EGTA, 0.2% (w/v) fatty acid free bovine serum albumin (BSA) and centrifuged at $2,000 \times g \times 4$ min. The supernatant was collected and centrifuged at $12,000 \times g \times 10$ min. The pellet was resuspended in MSEGTA and layered over 25% (v/v) Percoll/MSEGTA (100% Percoll solution contains 225 mM mannitol, 75 mM sucrose, 20 mM HEPES, 1 mM EGTA dissolved in 100% Percoll and adjusted to pH 7.4. This Percoll solution is diluted with MSEGTA mixture described above to 25% v/v) and centrifuged at $30,000 \times g \times 10$ min. Purified mitochondria fraction was collected at the bottom of the tube, resuspended in MSEGTA without added BSA and washed 2 times by centrifuging at $12,000 g \times 10$ min. Final mitochondrial pellet was resuspended in MS buffer comprising 225 mM mannitol, 75 mM sucrose, 20 mM HEPES-NaOH, pH 7.4 and stored on ice. Protein content was estimated by a commercial BCA assay (“Pierce Biotechnology”/“Thermo Scientific,” USA). Typically this procedure yields essentially pure, primarily non-synaptic, well-coupled mitochondria with respiratory control index 7–9 on glutamate plus malate.

2. Prepare the base buffer comprising 125 mM KCl, 4 mM KH₂PO₄, 14 mM NaCl, 20 mM HEPES-NaOH, pH 7.2, 1 mM MgCl₂, and 0.020 mM EGTA, in de-ionized water (Milli-Q, “Millipore”), adjust pH with KOH and sterilize the buffer by filtering it through 0.22 µm membrane. Stable for at least 3 months if kept sterile. Store at room temperature (or at +4°C) in an air-tight glass container. **DO NOT USE ANY PLASTIC VESSELS TO STORE THE BUFFER!** Avoid freezing the buffer. Prepare 1 ml of 100 mg/ml solution of bovine serum albumin, essentially fatty acids free, dissolved in de-ionized water (Milli-Q, “Millipore”); store frozen at –20°C. This can be frozen and thawed many times. Reconstitute the complete incubation buffer mixture right before the experiments; discard unused buffer after the experiment.
 - a. Note that the composition of our incubation buffer mimics the ionic composition of cell cytosol and allows one to study various and many mitochondrial functional activities under standard, directly comparable experimental conditions. Bovine serum albumin and EGTA are added to increase the degree of coupling of mitochondria by removing loosely bound long chain fatty acids and residual calcium contaminating other chemicals, respectively. Although the composition of incubation buffer can affect the measurements of H₂O₂, we have had good experience in using various other buffers that are commonly used in experiments with isolated mitochondria. For example, simplest iso-osmotic buffer suitable for H₂O₂ measurement can be composed of 225 mM mannitol, 75 mM sucrose, and 20 mM HEPES-NaOH (pH 7.4). Basically, the choice of a buffer should suit the experimental needs of the study. It is, however, strongly advised to avoid including reducing sugars such as glucose, bicarbonate, and compounds that are substrates or inhibitors of horseradish peroxidase. An optimal pH range for these measurements is 7.2–7.6.
3. Prepare 10 mM stock solution by adding 330 µl of anhydrous DMSO to the content of 1 mg package. Wrap in foil to protect from light. Aliquot by 10–20 µl and keep frozen at –80°C; an aliquot can normally be thawed-re-frozen once or twice. Discard when a faint pink color appears.
4. Prepare the stock solution by dissolving 1,000 U of horseradish peroxidase in 1 ml of de-ionized water (Milli-Q, “Millipore”). Aliquot by 20–50 µl and keep frozen at –80°C; discard unused solution after the experiment (do not re-use).
5. For all aliquoted reagents, use only non-colored, clear plastic tubes (e.g. PCR tubes or Eppendorf™ type 1.7 ml tubes), or – whenever possible – glass tubes.
6. To note, de-energized mitochondria do not routinely produce ROS. It is therefore suggested to have ready at least some common mitochondrial respiratory substrates and respiratory chain inhibitors, although the exact set depends on the purpose and the design of the ROS assay. In either case, some standard substrates and inhibitors can come up handy, such as (stock solutions) 1 M sodium glutamate, 1 M sodium malate, 1 M sodium succinate, all prepared in de-ionized water; also 1 mg/ml Antimycin A and 1 mM rotenone, prepared in absolute ethanol. These reagents can be frozen-thawed multiple times.
7. Prepare small amount (~250 µl) of ~40,000 U/ml stock solution in de-ionized water (Milli-Q, “Millipore”) right before the experiment. Do not freeze; keep on ice protected from light. Discard unused solution after the experiment.
8. Prepare 1 M solution in de-ionized water (Milli-Q, “Millipore”). Store frozen.

9. Prepare the stock solution by dissolving 10,000 U of superoxide dismutase in 1 ml of de-ionized water (Milli-Q, “Millipore”). Aliquot by 20–40 μ l and keep frozen at -80°C ; discard unused solution after the experiment (do not re-use).
10. In theory, a spectrophotometer can be used to detect ROS, as the compound produced in peroxidase catalyzed oxidation of Amplex Red Ultra by H_2O_2 has large absorption coefficient (please contact “Invitrogen” company for details). To the best of our knowledge, there were no publications where mitochondrial ROS were detected with Amplex Red Ultra spectro-photometrically. In either case, all the steps, reagents, and other information presented in this manuscript remain relevant irrespective of whether a fluorimeter or a spectrophotometer is used to detect ROS.
11. DO NOT USE METAL-CONTAINING DEVICES (e.g. Hamilton syringes with steel needles) to handle H_2O_2 solutions, substrate solutions, inhibitors, mitochondria or other liquids.
12. Superoxide dismutase is generally not required with experimental protocols resembling the one presented on Fig. 2. However, supplementing the incubation buffer with superoxide dismutase is recommended when it contains NAD(P)H or other compounds capable of superoxide-initiated, peroxidase-dependent redox cycling. In such conditions, superoxide dismutase will greatly improve the reproducibility and sensitivity of H_2O_2 assay. Considering that superoxide dismutase is a relatively cheap reagent, and that it does not negatively affect the assay sensitivity and reproducibility, we recommend routinely adding it to the assay buffer.
13. Do not keep the tube containing the incubation buffer anywhere close to the ventilation exhaust of a fluorimeter’s light source compartment. It will increase the residual concentration of H_2O_2 in the buffer and the baseline fluorescence.
14. Although pyruvate and alpha-ketoglutarate have recently gained some attention as “scavengers” of H_2O_2 in cellular systems (41–45), these substrates do not normally interfere with H_2O_2 measurements. Chemical decarboxylation of alpha-keto acids by H_2O_2 was reported more than a century ago (46); however, according to our experience and other published reports (45) the reaction of pyruvate and alphaketoglutarate with H_2O_2 is very slow particularly at low concentrations of H_2O_2 : $\sim 15\ \mu\text{M}$ H_2O_2 was not decomposed by 2 mM pyruvate in over 20 min at 37°C (45). Therefore, alpha-keto acids cannot compete for H_2O_2 with other targets such as our horseradish peroxidase-based H_2O_2 detection system or other intramitochondrial dehydrogenases.

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Fig. 1.
Calibrating the H_2O_2 assay. See text for the details.

Fig. 2. H_2O_2 emission by mouse brain mitochondria. Incubation medium (37°C) (Subheading 4.2) is supplemented with 4 U/ml horseradish peroxidase, 40 U/ml superoxide dismutase, 0.010 mM Amplex Red, and respiratory substrates as indicated. **(a)** Mouse brain mitochondria oxidizing succinate. **(b)** Mouse brain mitochondria oxidizing pyruvate and malate. Additions: Mito, 0.05 mg/ml mouse brain mitochondria; Succinate, 10 mM; Pyruvate:malate, 5:1 mM; Rotenone, 0.5 μ M; Antimycin, 1 μ g/ml. Numbers near the tracings are the rates of H_2O_2 production expressed in pmol/min/mg of mitochondria protein.