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Isolated respiring heart mitochondria release reactive oxygen species in states 4 and 3

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Abstract

Isolated mitochondria respiring on physiological substrates, both in state 4 and 3, are reported to be or not to be a source of reactive oxygen species (ROS). The cause of these discrepancies has been investigated. As protein concentration was raised in *in vitro* assays at 37°C, the rate of H_2O_2 release by rat heart mitochondria supplemented with pyruvate/malate or with succinate (plus rotenone) was shown to increase (0.03–0.15 mg protein/ml), to decrease (0.2–0.5 mg protein/ml) and to be negligible (over 0.5 mg protein/ml). The inhibition of mitochondrial respiration (with rotenone or antimycin A) or the increase in the oxygen concentration dissolved in the assay medium allowed an enhancement of ROS production rate throughout the studied range of protein concentrations. In mitochondria respiring in state 3 on pyruvate/malate or on succinate (plus rotenone), ROS release vanished for protein concentrations over 0.5 or 0.2 mg/ml, respectively. However, ROS production rates measured with low protein concentrations (below 0.1 mg/ml) or in oxygen-enriched media were similar or even slightly higher in the active respiratory state 3 than in the resting state 4 for both substrates. Consequently, these findings indicate that isolated mitochondria, respiring *in vitro* under conditions of forward electron transport, release ROS with Complex I- and II-linked substrates in the resting condition (state 4) and when energy demand is maximal (state 3), provided that there is sufficient oxygen dissolved in the medium.

Keywords: *Mitochondrial respiratory state, free radicals, hydrogen peroxide (H_2O_2), protein concentration, oxygen*

Abbreviations: FAF-BSA, fatty acid-free bovine serum albumin; HRP, horseradish peroxidase; HVA, homovanillic acid; ROS, reactive oxygen species; RCI, respiratory control index; SOD, superoxide dismutase

Introduction

Reactive oxygen species (ROS) are considered as inevitable by-products of normal aerobic metabolism. Most *in vitro* studies indicate that the main source of ROS in animal cells is the mitochondria [1,2]. The mitochondrial electron transport chain contains several redox centres that may leak electrons to oxygen, generating superoxide anion ($O_2^{\cdot-}$), the primary ROS [3]. Although, superoxide is a radical that cannot cross membranes, a large portion of $O_2^{\cdot-}$ was reported to exit mitochondria via voltage-dependent anion channels [4]. Dismutation of superoxide, spontaneously or catalysed by superoxide dismutase (SOD), produces hydrogen peroxide (H_2O_2), which in turn may be reduced or can diffuse rapidly through membranes and reach the exterior of the mitochondria. Therefore, the net release of ROS from mitochondria to cytosol reflects the balance between ROS production and ROS scavenging inside the organelle.

Although mitochondrial ROS production is readily observed *in vitro* [1,3], there is some controversy as to whether mitochondria are an important source

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of ROS under physiological conditions [5–7]. It is unequivocally accepted that isolated respiring mitochondria or submitochondrial particles can be compelled to generate ROS when inhibitors of the electron transport chain, specially antimycin A for Complex III and rotenone for Complex I, are present [1,4,8]. Likewise, mitochondria respiring on succinate, the substrate for Complex II, in the absence of rotenone exhibit high rates of ROS production [9–12], mostly due to reverse electron transport into Complex I. The physiological relevance of both situations, inhibition of the electron transport chain and reverse electron flow, is unclear.

In contrast, the forward electron transport through the respiratory chain, associated with mitochondria respiring in state 4 on Complex I-linked substrates or on succinate in the presence of rotenone, is clearly physiological. Under these conditions, isolated mitochondria generate ROS at relatively lower rates and conflicting results exist in the literature. Thus, either mitochondria are reported not to release measurable amounts of H_2O_2 [6,13], or basal rates of ROS production are detected [4,10,14,15], rates that depend on the tissue and the respiratory substrate employed. In some studies, H_2O_2 release in the presence of NADH-linked substrates was barely detectable, whereas succinate (plus rotenone)-supported respiration resulted in measurable rates of ROS release [10,11]; other investigations described comparable levels of mitochondrial H_2O_2 production in the presence of Complex I-linked substrates or succinate [1,16,17].

Among the physiological factors that regulate mitochondrial ROS generation, it is of particular interest the metabolic state, which may be assessed *in vitro* by the respiratory state of mitochondria. Thus, *in vivo* muscle mitochondria can experience periods of resting activity, when they may be close to the state 4 respiration rate, but upon initiation of contraction, the ATP demand and raised Ca^{2+} may be such as to cause transition to state 3 [18]. There is no consensus about the ability of mitochondria to release measurable amounts of ROS in state 3. Early studies found that the addition of ADP to cause the transition from the resting state 4 to the active state 3 stopped H_2O_2 production by heart mitochondria respiring on succinate in the absence of rotenone; after added ADP was phosphorylated (state 3–4 transition), H_2O_2 release accelerated to the previous rate [9,19]. The finding led to the widespread notion that mitochondria only produce ROS under the resting state 4 conditions. Subsequently, H_2O_2 production on Complex I-linked substrates was reported to be independent of mitochondrial respiratory state or even to increase when the respiration switched from the resting to the active state in heart and brain mitochondria [20], whereas ADP stimulated twofold H_2O_2 formation by diaphragm mitochondria [21]. In contrast, in limb skeletal muscle mitochondria, H_2O_2 release was 50 and 20% lower in state 3 than in state 4 when succinate (plus rotenone) or pyruvate/malate, respectively, were used as substrates [16], and in brain mitochondria respiring on NADH-linked substrates ROS release rate was reported to be reduced by approximately 70% during state 3 respiration [22]. The reason of these apparent discrepancies is unknown at present.

This study was aimed at exploring the ability to generate ROS of intact isolated mitochondria respiring *in vitro* under physiologically relevant experimental conditions. H_2O_2 release rates were measured during forward electron transport through the respiratory chain using rat heart mitochondria energized with succinate (in the presence of rotenone) or with pyruvate/malate, either in resting state 4 or in active state 3. Our results indicate that respiring mitochondria are a reliable source of ROS, and that protein concentration and oxygen availability in the assay medium are important factors that determine the values of the measured rates of ROS release by mitochondria.

Materials and methods

Chemicals and biochemicals

Essentially fatty acid-free bovine serum albumin (FAF-BSA), Nagarse (bacterial proteinase, type XXIV), homovanillic acid (HVA, 3-methoxy-4-hydroxyphenylacetic acid), SOD (from bovine erythrocytes), rotenone and antimycin A were purchased from Sigma-Aldrich (Spain). Horseradish peroxidase (HRP, grade II) was from Roche (Boehringer, Spain). Other chemicals came from Sigma or Merck.

Isolation of mitochondria

Heart mitochondria were prepared from male Wistar rats, weighing 300–400 g, obtained from the colony maintained at the Animal Facility of the Biology Faculty, Complutense University. Animals were decapitated and the heart was quickly excised and plunged into ice-cold isolation buffer. Vessels and auricles as well as remaining blood were removed and the tissue was chopped into small pieces with scissors. The isolation of mitochondria was performed at 4°C as quick as possible and according to Barja [23]. Tissue was homogenized with a loose-fitting pestle in 10 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4) containing in addition 5 mg of Nagarse and 25 mg of FAF-BSA. After standing for 1 min, 25 ml of additional isolation buffer containing 25 mg of FAF-BSA were added and homogenisation was gently performed again with a tighter-fitting pestle. After homogenisation, the pH was checked and adjusted to 7.4 if needed. The homogenate was centrifuged at

700*g* for 10 min. The mitochondria were obtained after centrifugation of the first supernatant at 8000*g* for 10 min. The second supernatant is discarded together with the fluffy layer on top of the mitochondrial light-brown pellet found at the bottom. To wash the mitochondria, the pellet was resuspended in 20 ml of isolation buffer and centrifuged at 8000*g* for 10 min. Finally, the mitochondrial pellet was resuspended in 1 ml of isolation medium and kept on ice to use immediately for the oxygen consumption and ROS release measurements. Mitochondrial protein was determined in the final suspension by the Lowry method [24].

Mitochondrial oxygen consumption

Mitochondrial respiration was measured polarographically at 37°C with a computer-controlled Clark-type O_2 electrode (Oxygraph, Hansatech, Norfolk, UK) in 0.5 ml of respiratory buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% FAF-BSA, pH 7.4) with 0.25 mg of mitochondrial protein per ml and succinate (5 mM, plus 2 μM rotenone) or pyruvate/malate (2.5 mM/2.5 mM) as substrates, in the absence (state 4) followed by in the presence (state 3) of 500 μM ADP. The respiratory control index (RCI) was calculated as state 3/state 4 oxygen consumption ratio. RCI values of 4.8 ± 0.4 and 2.2 ± 0.2 were obtained with pyruvate/malate and succinate plus rotenone, respectively, indicating a suitable degree of integrity of the mitochondrial preparations.

Mitochondrial ROS release

The rate of mitochondrial ROS release was assayed at 37°C by measuring the linear increase in fluorescence caused by oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase [23,25]. Fluorescence measurements were performed using a computer-controlled Aminco-Bowman Series 2 luminescence spectrometer, equipped with a thermostatted and magnetic-stirred cell sample holder and with a microsampling accessory allowing external additions during the scans. Kinetic assays and fluorescence measurements were performed in 3 ml fluorimeter quartz cuvettes (excitation at 312 nm, emission at 420 nm, slits 4 and 8 nm, respectively). Reaction conditions were the indicated concentration of mitochondrial protein, 6 U/ml of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of SOD and substrate, in a total volume of 1.5 ml of the same respiratory buffer used for O_2 consumption. Production of ROS was started by adding the substrate: 2.5 mM pyruvate and 2.5 mM malate or 5 mM succinate plus 2 μM rotenone (included to avoid the backward flow of electrons from Complex II to I). In some experiments ADP (500 μM), rotenone (2 μM) or antimycin A (10 μM) were also included in the reaction mixture.

In the kinetic assays, the reaction rate was continuously followed at pH 7.4 and 37°C for a few minutes and the slope (arbitrary fluorescence units per min) was determined by the computer software. In the fixed-time assays, after 15 min of incubation at pH 7.4 and 37°C in a water bath with shaking, the reaction was stopped by transferring the samples to an ice-cold bath and addition of 0.5 ml of 0.1 M glycine–NaOH, pH 12, containing 25 mM EDTA (glycine–EDTA) [25,26] and, subsequently, the fluorescence was determined at 25°C and pH 10. Fixed-time assays were performed at different oxygen availabilities, achieved by carrying out the assay either in open test tubes with air-saturated buffer (type I), or in sealed flasks gassed during the assay with oxygen (100% O_2) (type II), or in open tubes where the medium was continuously bubbled with oxygen (type III); oxygen concentration was determined by polarographic analysis of the assay media under experimental conditions analogous to those of ROS release experiments.

Appropriate blanks, with all the reaction components but without substrate, were run in parallel to correct the fluorescence of the samples. Known concentrations of H_2O_2 (molar extinction coefficient at 230 nm, 71 $M^{-1}\,cm^{-1}$) were used to convert fluorescence units to nanomoles of H_2O_2 that were referred to min and to mg of mitochondrial protein. Due to the strong pH-dependence of the fluorescence intensity of HVA oxidation product, independent H_2O_2 standard curves were established for each assay type (not shown). It was verified that the time-course of H_2O_2 release by mitochondria (0.1 mg protein/ml) was linear and that kinetic and fixed-time assays led to comparable results (not shown); inter-assay variations were usually within 10–15%. Under the described experimental conditions (assay medium containing homovanillic acid, HRP and SOD), no significant rates of apparent H_2O_2 production were observed in the presence of substrates or inhibitors when mitochondria were absent. The sensitivity of the method (a minimum rate of 10 pmol/min/ml can be determined reliably) allows the measurement of H_2O_2 release by isolated mitochondria that respired in the absence of respiratory chain inhibitors (see below).

The presence in the assay mixture of low concentrations of mitochondria (up to 0.1 mg protein/ml) did not modify significantly the HVA-HRP fluorescence increases caused by known concentrations of H_2O_2; these results indicate that with low mitochondria concentrations, the employed concentrations of HVA (0.1 mM) and HRP (6 U/ml) circumvent interference by competing endogenous hydrogen donors present in the biological sample and by intra-mitochondrial enzymes that actively metabolise H_2O_2, respectively. However, higher

mitochondria concentrations quenched the fluorescence: the slopes of the H_2O_2 standard curves obtained in the presence of 0.2, 0.4 and 0.6 mg of mitochondrial protein per ml were 90, 80 and 75%, respectively, with regard to the slope of the control curve without mitochondria. Therefore, in this study, the rates of mitochondrial H_2O_2 production were calculated using the corresponding H_2O_2 standard curve obtained in the presence of the concentration of mitochondria employed in the assay.

We measured the rates of H_2O_2 release by isolated mitochondria in the presence of exogenous SOD. The addition of SOD (50 U/ml) did not modified the standard curves for H_2O_2. However, when respiring mitochondria were present in the assay, added SOD caused a significant increase in the fluorescence signal (50–55% with pyruvate/malate or succinate plus rotenone), suggesting that with both substrates mitochondria release $O_2^{\cdot -}$ in addition to H_2O_2, what has been previously proposed [4,13]. SOD was in excess in the experiments since doubling the concentration of the enzyme did not affect the results. Therefore, the assay employed in this study, performed in the presence of exogenous SOD, estimates the mitochondrial release of H_2O_2 plus $O_2^{\cdot -}$, referred to as ROS release.

Statistical analyses

Results are expressed as means ± SD in tables and figures. SD is indicated by verticals bars in figures. Assays were always performed in duplicate, with n independent mitochondrial preparations. Differences between means in the absence or in the presence of ADP for ROS release rate and for the percentage of total consumed oxygen released as H_2O_2 were statistically analysed with Student's t tests. Probability values ($P < 0.05$) were considered significant.

Results

Relationship between ROS release rate and mitochondria concentration

Superoxide anion formation by the mitochondrial respiratory chain is a non-enzymatic process, the rate of which is controlled primarily by mass action [3], increasing when the concentration of electron donors ($R^{\cdot}$) or of molecular oxygen rises:

$$R^{\cdot} + O_2 \rightarrow R + O_2^{\cdot -}$$

$$d[O_2^{\cdot -}]/dt = k[O_2][R^{\cdot}]$$

Hence, *in vitro* under established experimental conditions and at a fixed oxygen concentration, the initial rate of the reaction is expected to be proportional to the concentration of mitochondria (i.e. electron donors) in the assay. Surprisingly, as Figure 1 shows, in the protein concentration range usually employed in the literature (0.1–0.5 mg/ml), there was not a linear relationship between ROS release rates and the concentration of mitochondria respiring on substrates linked to Complex I (pyruvate/malate) or complex II (succinate plus rotenone). In contrast, the curves exhibited successively a rise (for low protein concentrations), a peak (corresponding to 0.1 and 0.2 mg protein/ml for pyruvate/malate and succinate plus rotenone, respectively) and a steady decline (for higher concentrations of mitochondria).

Two common causes may give rise to a maximum or even a decrease in the reaction rate when increasing the concentration of protein. The first possibility is a failure to measure the true initial rate of the reaction. Since, kinetic assays were performed, the time-course of product formation was directly obtained from the fluorimeter, and the H_2O_2 liberation rate was calculated from the initial linear part of the reaction progress curve. In the second place, at high protein concentrations, the detection method may become rate-limiting. It was verified that a 20-fold increase in HVA concentration (2 mM) in the assay did not avoid the anomalous behaviour. Neither was the curve normalized if the final concentrations of HRP or substrates were doubled. Taking into account, the excitation and emission wavelengths for HVA, a third possibility may be that the incident light beam and the light emitted by the excited HVA product were partially absorbed by the mitochondrial constituents in the fluorimeter cuvette. As mentioned above, the use of H_2O_2 standard curves

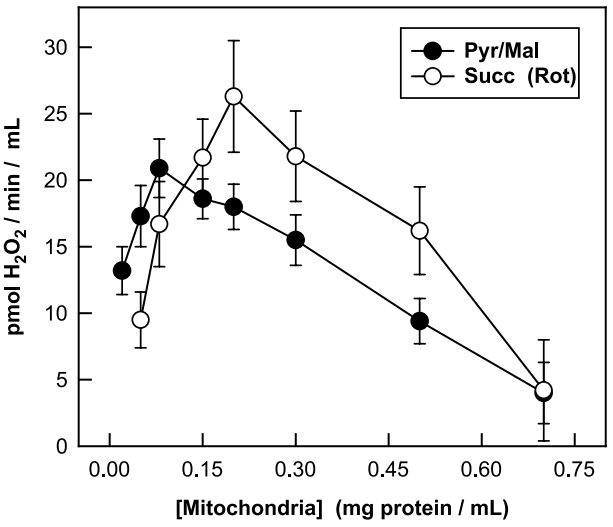

Figure 1. Effect of mitochondria concentration on the rate of ROS release by rat heart mitochondria respiring in state 4. H_2O_2 release was measured employing the kinetic assay with the indicated concentrations of mitochondrial protein and 2.5 mM pyruvate/2.5 mM malate (•) or 5 mM succinate (plus 2 μM rotenone) (○) as substrates. Data represent means ± SD of 4–6 independent mitochondrial preparations.

calibrated with mitochondria compensated for this interference to the detection method.

We tested whether different agents succeeded in avoiding the decrease in ROS release rate observed when increasing mitochondria concentration. The addition of the chaotropic agent thiocyanate (SCN^-) to the assay medium containing pyruvate/malate or succinate (plus rotenone) improved the curve profile (Figure 2A): thiocyanate elicited an enhancement of H_2O_2 release rate with protein concentration and almost abolished mitochondrial oxygen consumption (not shown). Likewise, ROS release rate increased with mitochondrial protein concentration when antimycin A or rotenone, classical inhibitors of the respiratory chain, were added to the assay (Figure 2B). These results suggest that the described anomalous behaviour disappears when oxygen consumption by mitochondria is markedly inhibited. Since, oxygen is a necessary substrate for superoxide generation, the observed decreases in H_2O_2 liberation rate with high mitochondria concentrations may be associated with concomitant descents of oxygen concentration in the assay medium due to mitochondrial respiration.

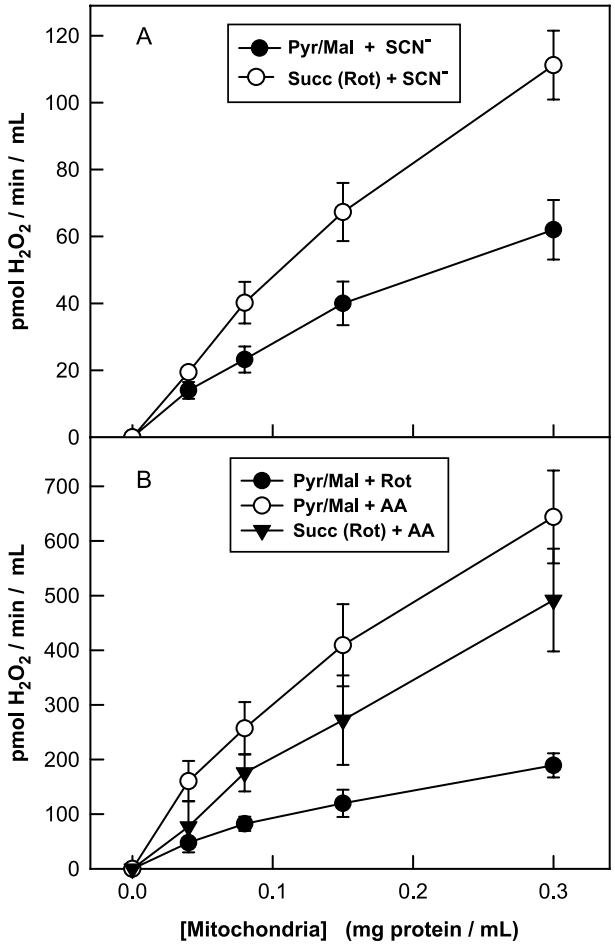

Figure 2. Effect of mitochondria concentration on the rate of ROS release by rat heart mitochondria incubated in the presence of substrate and thiocyanate (**A**) or substrate and inhibitors of the respiratory electron transport chain (**B**). H_2O_2 release was measured employing the kinetic assay with the indicated concentrations of mitochondrial protein and with pyruvate/malate (Pyr/Mal) or succinate (plus rotenone) (Succ (Rot)) as substrates. When present, concentration of reactants was as follows: 0.25 M thiocyanate (SCN^-), 2 μM rotenone (Rot), 10 μM antimycin A (AA). Data represent means ± SD of 3 independent mitochondrial preparations.

Effect of oxygen availability on ROS release rate

To verify the proposed hypothesis, the effect of respiring mitochondria on the concentration of oxygen dissolved in the assay medium was determined (Figure 3). The experimental conditions employed were analogous to those of the kinetic assay for ROS release, i.e. an air-equilibrated medium with the same concentrations of chemicals and mitochondria, the same incubation temperature and magnetic stirring rate, whereas the air-open surface of the liquid was similar (0.8 cm^2 and round for the oxygen electrode chamber and 1 cm^2 and square for the fluorimeter cuvette). As expected, the higher the mitochondria concentration was, the quicker the concentration of oxygen dissolved in the assay medium declined, oxygen concentration that, in some cases, was fully abolished. For a fixed mitochondria concentration, the higher was the respiration rate (succinate vs pyruvate/malate; in the presence vs in the absence of ADP), the larger and quicker was the descent in oxygen concentration.

On the other hand, ROS release assays were performed at different oxygen availabilities (initial oxygen concentration in the assay solution was 0.2, 0.8 and 1.0 mM for type I, II and III assays, respectively). As Figure 4 shows, when oxygen concentration in the assay medium was elevated (type II and III assays), ROS production rates showed a steady rise with the concentration of respiring mitochondria. The profiles obtained for mitochondria supplemented with pyruvate/malate or with succinate (plus rotenone) were alike, showing an open hyperbolic dependence on mitochondria concentration; below 0.1 mg protein/ml the increase in mitochondrial ROS production with protein concentration can be regarded as linear. In agreement with previously published results [27,28], for a fixed concentration of mitochondria, ROS release rate increased with oxygen concentration in the assay medium. Therefore, the downward trend in the curve that relates ROS release rate and mitochondria concentration was reversed in the presence of high concentrations of oxygen.

Mitochondrial ROS release rate during state 3 respiration

Taking into account that in coupled mitochondria, the transition from respiratory state 4 to 3 increases several-fold the oxygen consumption rate, the addition of ADP into the ROS release assay might enhance the

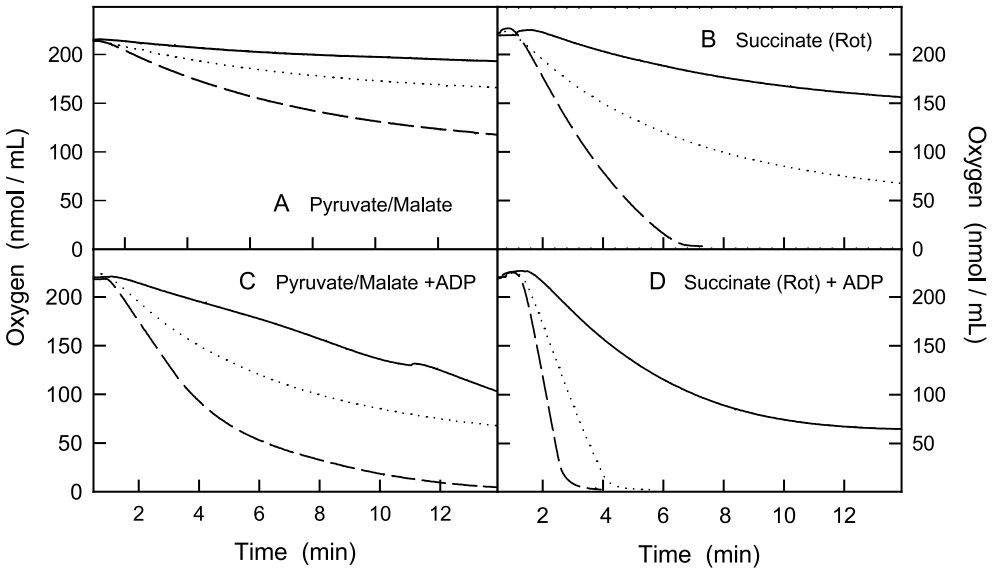

Figure 3. Effect of mitochondria concentration on the rate of consumption of dissolved oxygen by respiring rat heart mitochondria in an air-open reaction vessel. Representative oxygraphic traces of mitochondria respiring in air-equilibrated respiration buffer at 37°C on 2.5 mM pyruvate/ 2.5 mM malate (A and C) or 5 mM succinate (plus 2 μM rotenone) (B and D), either in the absence (A and B) or in the presence (C and D) of 0.5 mM ADP. Mitochondrial protein concentrations: 0.08 mg/mL (continuous line), 0.25 mg/mL (dotted line), 0.5 mg/mL (dashed line). Oxygen concentration was determined by polarographic analysis using an oxygen electrode (Oxygraph, Hansatech Ltd.) in which the reaction chamber was not closed with the adjustable stopper, but left air-open. The reaction volume was 1.3 mL (liquid height 1.6 cm, open surface 0.8 cm^2) and it was constantly stirred during the measurements.

anomalous behaviour that, with respect to protein concentration, was observed in air-saturated medium. However, in mitochondria supplemented with pyruvate/malate (Figure 5), the rates of ROS production were not significantly different during state 3 and 4 respiration under the different experimental conditions employed (protein concentrations and oxygen availabilities). It is worth pointing out that in air-saturated medium (Figure 5A), at low protein concentrations (up to 0.1 mg/ml), the rate of ROS release was slightly higher in the presence than in the absence of ADP. These results are consistent with previously reported observations, performed in air-saturated media with Complex I-linked substrates, where mitochondrial H_2O_2 production rates in state 3 were not abolished, but they were found to be higher [21], similar [20], or lower [15,16] than those measured during resting respiration.

On the other hand, with succinate (plus rotenone) as substrate (Figure 6), respiration transition from state 4 to 3 elicited remarkable changes in mitochondrial ROS release rate. In air-equilibrated medium (Figure 6A), the commonly employed condition for *in vitro* assays, with protein concentrations over 0.2 mg/ml, addition of ADP practically stopped ROS production, in agreement with the classically reported results for H_2O_2 release by mitochondria respiring on succinate [9,19,20,29]. Nevertheless, when the concentration of protein was lower (below 0.1 mg/ml) and hence, the available oxygen in the assay medium were only partially consumed by mitochondria, and in the presence of rotenone, to avoid reverse electron transfer from succinate, mitochondria respiring in state 3 released ROS at slightly higher rates than in state 4 (Figure 6A). Likewise, when ROS release assays were performed in the presence of elevated oxygen concentrations, the described halt in ROS production associated with high mitochondria concentrations vanished, and the rates of ROS release were similar (Figure 6B) or even higher (Figure 6C) during active state 3 than during resting state 4 respiration.

The rates of ROS release and oxygen consumption were measured in air-equilibrated medium under the same experimental conditions (buffer composition, concentrations of substrates and ADP, assay temperature), allowing the calculation of the oxygen fraction turned into ROS instead of being reduced to water (Table I). Such a fraction was higher when mitochondria were supplemented with pyruvate/malate than with succinate (plus rotenone), and before (state 4) than after ADP addition (state 3), with both kinds of substrates; the described differences mainly came from the changes produced in oxygen consumption. These results and previous studies [15,16,20] indicate that mitochondrial ROS release does not increase in parallel to mitochondrial respiration rate.

Discussion

Our results show that the measured rates of ROS release by isolated mitochondria respiring under physiologically relevant conditions are strongly affected by the mitochondria concentration employed

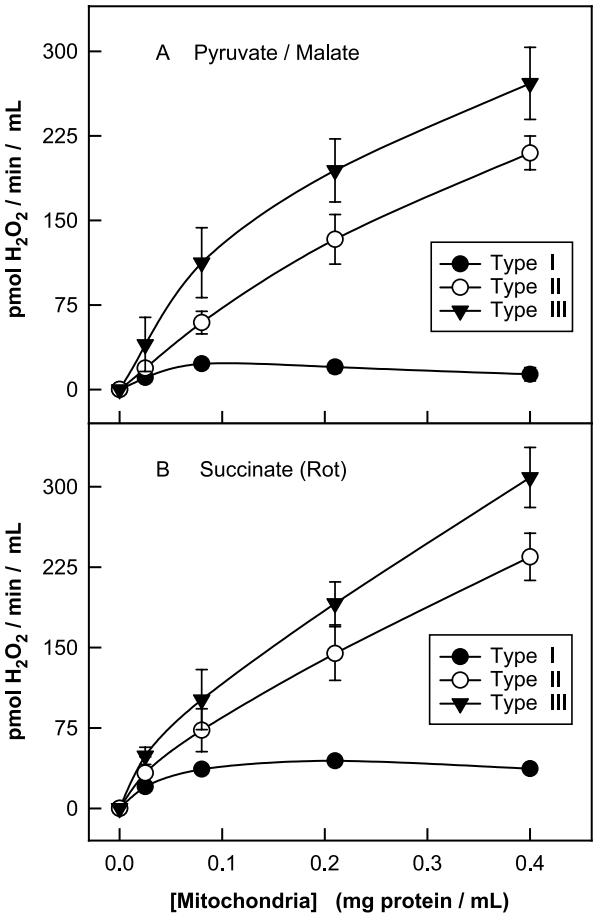

Figure 4. Effect of oxygen availability on the rate of ROS release by rat heart mitochondria respiring on pyruvate/malate (**A**) or succinate (plus rotenone) (**B**). To measure H_2O_2 release, fixed-time assays (15 min of incubation at 37°C with shaking) were performed with the indicated concentrations of mitochondrial protein either in open test tubes (type I, •), or in sealed flasks gassed with oxygen (type II, ○), or in open tubes where the medium was continuously bubbled with oxygen (type III, ▼). Initial oxygen concentration in the assay solution was 0.2, 0.8 and 1.0 mM for type I, type II and type III assays, respectively. Data represent means ± SD of 3 independent mitochondrial preparations.

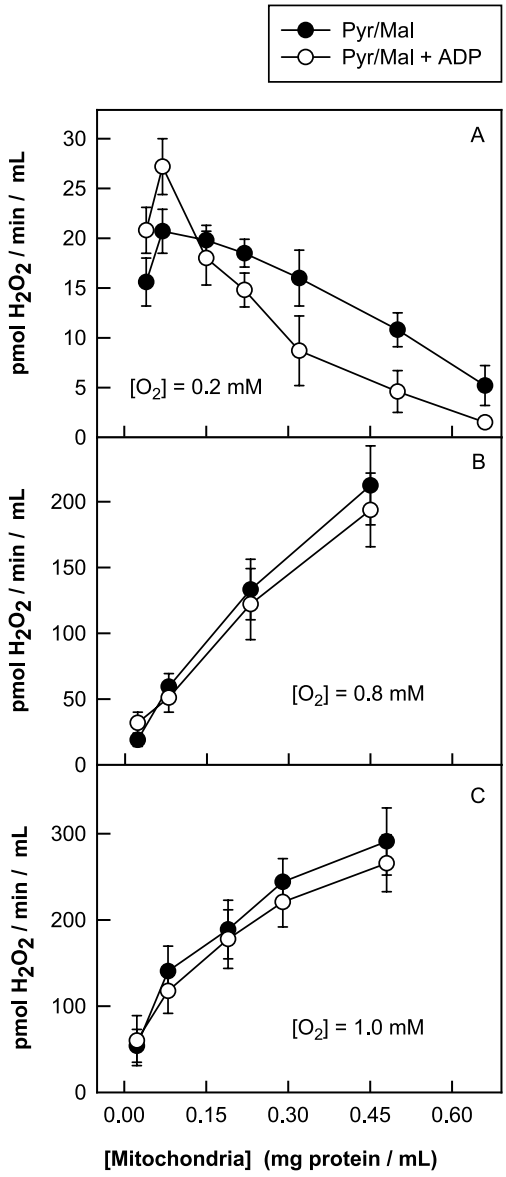

Figure 5. Effect of ADP and oxygen availability on the rate of ROS release by rat heart mitochondria respiring on pyruvate/malate. To measure H_2O_2 release, assays were carried out with the indicated concentrations of mitochondrial protein incubated with 2.5 mM pyruvate/2.5 mM malate either in the absence (state 4) (•) or in the presence (state 3) of 0.5 mM ADP (○). Initial oxygen concentration in the assay medium: **A**, 0.2 mM (kinetic assays); **B**, 0.8 mM (fixed-time assays of type II); **C**, 1.0 mM (fixed-time assays of type III). Data represent means ± SD of 3 independent mitochondrial preparations.

in the *in vitro* assay. This fact may be attributed, at least in part, to the consumption of the dissolved oxygen available in the assay medium. Oxygen is the shared substrate for the enzymatic (respiration) and non-enzymatic (ROS production) reactions of O_2 reduction. It is well-known that cytochrome oxidase exhibits an extremely high affinity to O_2 ($K_M < 10^{-6}$ M), whereas the one-electron reduction proceeds without any specific oxygen-binding site involved [30]. Thus, at standard dissolved oxygen concentration for *in vitro* conditions (0.2 mM at 37°C), enzymatic O_2 reduction is over-saturated, but $O_2^{\cdot-}$ formation is considered to be a linear function of oxygen concentration. In addition, the rate of enzymatic oxygen consumption is about two orders of magnitude higher than that of $O_2^{\cdot-}$ formation [30, Table I]. Taking into account that the amount of dissolved oxygen present in the assay medium is limited (even when, to facilitate O_2 diffusion from air, the solution were kept open and constantly stirred), the higher the concentration of respiring mitochondria employed, the quicker and deeper the decay of oxygen in solution is. The concentration of oxygen dissolved in the assay medium will reflect the balance between the rate of diffusion of O_2 from air into the medium and the rate of oxygen consumption reactions, which depends mainly on mitochondria concentration and

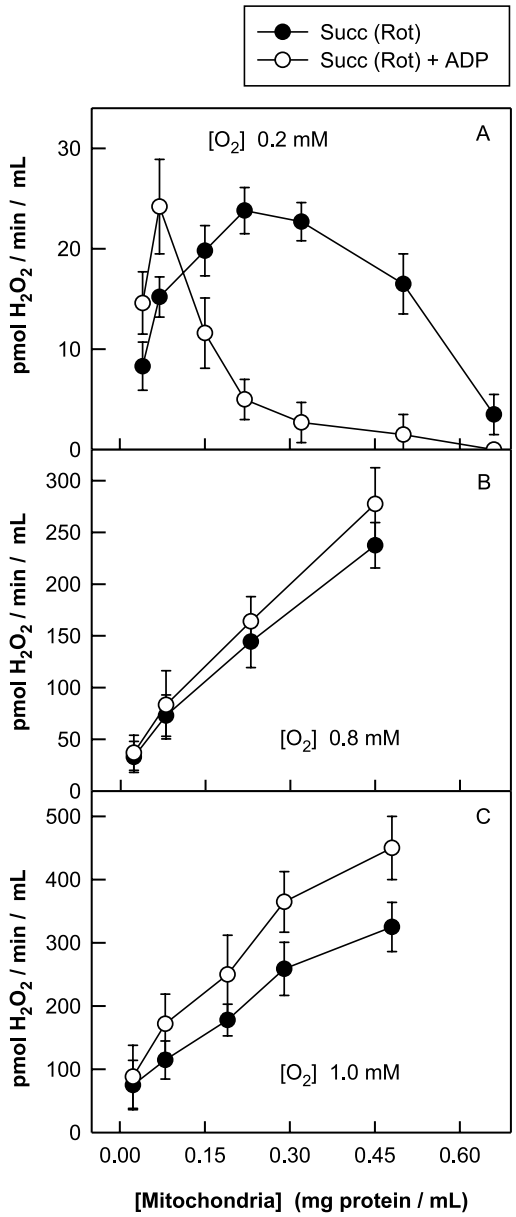

Figure 6. Effect of ADP and oxygen availability on the rate of ROS release by rat heart mitochondria respiring on succinate (plus rotenone). To measure H_2O_2 release, assays were carried out with the indicated concentrations of mitochondrial protein incubated with 5 mM succinate (plus 2 μM rotenone) either in the absence (state 4) (•) or in the presence (state 3) of 0.5 mM ADP (○). Initial oxygen concentration in the assay medium: **A**, 0.2 mM (kinetic assays); **B**, 0.8 mM (fixed-time assays of type II); **C**, 1.0 mM (fixed-time assays of type III). Data represent means ± SD of 3 independent mitochondrial preparations.

their respiratory activity, and on assay temperature. Thus, for elevated mitochondria concentrations or when respiration rate is high (i.e. state 3), the rate of dissolution of oxygen from air may be insufficient to compensate the rate of dissolved oxygen consumption and, consequently, the concentration of oxygen in the medium will decrease (see Figure 3). The drop in oxygen concentration of the medium may, in turn, elicit a decrease in ROS formation rate, without modifying the respiration rate until O_2 concentration is low enough to desaturate the cytochrome oxidase (less than 10 μM). This may contribute to explain why the measured mitochondrial ROS release rates were dependent on the concentration and the respiration rate of mitochondria, being higher or lower as a function of the relative oxygen concentration established in the assay medium.

In general, protocols for the determination of mitochondrial ROS release suggest the use of a fixed [6,23] or of a small range of protein concentrations [31]. Surprisingly, and to the best of our knowledge, the literature does not contain any explicit information about the effect on ROS release of the concentration of intact mitochondria respiring on physiological substrates, although there are some related results. Thus, H_2O_2 release rate was shown to increase linearly with mitochondria concentration when, in addition to substrate, antimycin A and an uncoupler were present [27]. Likewise, H_2O_2 production was found to be directly proportional to the number of cells in the assay, showing a plateau for high macrophage concentrations [25]. In both cases, oxygen consumption was limited either by the presence of a respiratory chain inhibitor or because cells consume less oxygen than isolated mitochondria. When mitochondria freely respiring on physiological substrates are considered, ROS release rates in air-saturated buffer (Figure 1) increase only with low mitochondria concentrations that do not modify greatly the concentration of dissolved oxygen; however, the inhibition of mitochondrial respiratory activity (Figure 2), or the use of oxygen-enriched media (Figure 4) allow higher mitochondria concentrations to be used without the appearance of a decrease in H_2O_2 production rate. The results of the present study underline the importance of using an appropriate concentration of respiring mitochondria for *in vitro* measurements of mitochondrial ROS release, and account, at least in part, for the reported differences on the mitochondrial ability to produce ROS under conditions of forward electron flow through the respiratory chain. Thus, the observations of H_2O_2 release by intact mitochondria respiring under physiologically relevant conditions correspond mostly to assays performed with low concentrations of protein (below 0.1 mg/ml) [14,17,21,27, this work]. In contrast, the studies that failed to show significant mitochondrial ROS production rates with Complex I- or II-linked substrates, employed higher protein concentrations (0.4–0.5 mg/ml) [6,13], experimental conditions that should lead to a fast consumption of the oxygen dissolved in the assay medium.

Yet another important factor about the *in vitro* determinations of ROS release rate is the level of respiratory activity which, in turn, depends on the metabolic state and the quality ("coupling") of

Table I. Respiration rate, ROS release rate and percentage of total consumed oxygen released as ROS by isolated rat heart mitochondria oxidizing Complex I- and Complex II-linked substrates in state 4 and state 3.

Substrate	Respiratory state	O_2 consumption (nmol O_2 min^{-1} mg $protein^{-1}$)	ROS release[a] (nmol H_2O_2 min^{-1} mg $protein^{-1}$)	% total O_2 consumed released as ROS[b]
Pyr/Mal	State 4	28.4 ± 5.5	0.25 ± 0.03	0.88 ± 0.10
	State 3	124.8 ± 13.1⋆	0.29 ± 0.04	0.24 ± 0.04⋆
Succ (Rot)	State 4	117.5 ± 18.7	0.19 ± 0.05	0.18 ± 0.04
	State 3	253.3 ± 32.3⋆	0.28 ± 0.07⋆	0.11 ± 0.04⋆

Values are means ± SD of at least 5 independent mitochondrial preparations. Pyr/Mal, 2.5 mM pyruvate/2.5 mM malate; Succ (Rot), 5 mM succinate plus 2 μM rotenone; state 3, with 500 μM ADP present.
⋆$P < 0.05$, significant difference compared to state 4; [a]ROS release rate measured employing the kinetic assay with 0.07–0.08 mg mitochondrial protein per mL; [b]nmol H_2O_2 released per nmol O_2 consumed, expressed as percentage.

mitochondria. Our results show that the energetic transition caused by ADP, from resting to active respiratory state, did not elicit decreases in the mitochondrial ROS production rates associated with forward electron transfer, provided there is sufficient oxygen dissolved in the medium. Relatively few data are available on mitochondrial H_2O_2 generation in phosphorylating or state 3 respiration. This is probably the result of the widespread belief that mitochondria only produce ROS in state 4, idea derived from the virtual stopping of H_2O_2 production after the addition of ADP to heart mitochondria respiring on succinate [9,19,20]; this finding has been interpreted as a mechanism of protection against ROS generation for mitochondria engaged in ATP synthesis. In recent years, it has become apparent that most of ROS production supported by succinate oxidation in resting state 4, which occurs via reverse electron transport into Complex I [12], is very sensitive to mitochondrial membrane potential ($\Delta\psi_m$) [10,11,29] or to pH gradient across the mitochondrial inner membrane [12]. Thus, even the small depolarisation associated with ATP synthesis (state 3 respiration) is sufficient to abolish H_2O_2 production [11,20,29]. Likewise, the presence in the assay of natural or artificial uncouplers (uncoupled state respiration) [11,32,33], or oddly of the Complex I inhibitor rotenone [10–12,33], strongly depresses ROS release by mitochondria respiring on succinate. These dramatic decreases in ROS production are accounted for by the impairment of reverse electron transport into Complex I [12,32,33].

Nevertheless, ROS production derived from forward electron flux through respiratory chain appears not to be so highly dependent on membrane potential [34], and other factors, such as the level of reduction of NAD(P)H mitochondrial pool, apparently regulate the rate of ROS release [22,35]. A significant fraction of H_2O_2 formation in the presence of NADH-linked substrates persisted following maximal uncoupling [22] or ADP addition [15–17], and in some cases, mitochondria in state 3 produced ROS at rates similar to even higher than in state 4 [20,21, this study]. When forward electron flux came from succinate in the presence of rotenone, ADP addition elicited decreases in ROS release rates with high concentrations of protein in the assay, probably related with the very high oxygen consumption; however, with low protein concentrations (up to 0.1 mg/ml), mitochondria in state 3 produced ROS at rates similar [this work] or lower, but significant [16,17], than during resting respiration. Consequently, it can be concluded that, under appropriate experimental conditions, i.e. forward electron transport and sufficient oxygen, isolated mitochondria *in vitro* release ROS with Complex I- and II-linked substrates in the respiratory state 3.

The limitations of *in vitro* studies must be kept in mind. On the one hand, a variety of structural and functional alterations associated with the mechanical isolation of mitochondria from their natural environment in the cell may lead to artefactual deviation of odd electrons to oxygen [36]. On the other hand, muscle mitochondria *in vivo* probably never experience the experimental conditions commonly employed *in vitro*. First, in the intracellular microenvironment, mitochondria are well separated from air-level oxygen pressure (equivalent to a dissolved oxygen concentration of 0.2 mM). The range of 10–30 μM is representative of tissue intracellular oxygen level, that is reported to be even lower (3–8 μM) in the heart [37]. Myoglobin, a mobile carrier of oxygen abundant in striated muscles that contract for long periods, facilitates the continuous oxygen delivery to the mitochondria that operate in sustained steady states of low oxygen pressure that resist change in response to variation in muscle work or oxygen supply. Therefore, the *in vivo* rate of mitochondrial ROS production must be, no doubt, considerably less than the estimates made on isolated mitochondria in the presence of non-physiological concentrations of oxygen. Second, isolated mitochondria lack the antioxidant systems of cytosol, although they possess the vigorous intramitochondrial antioxidant defences,

including enzymes (SOD, glutathione peroxidase, catalase, peroxiredoxin) and free radical scavengers (e.g. glutathione, cytochrome *c*, ubiquinol, vitamin E) [3]. Third, in spite of the fact that in muscle the rates of oxidative phosphorylation and ATP utilization vary widely with work load, *in vivo* conditions would not allow mitochondria to reach respiratory state 3 or 4. Mitochondria in cells are often described as being in a state intermediate between states 4 and 3 and, in continuously working aerobic tissues like the heart, the mitochondrial state is considered to be closer to state 3 than to state 4 [20].

In conclusion, these findings indicate the importance of the available oxygen concentration among the factors that control the rate of ROS production by respiring mitochondria. The variability among laboratories about ROS release by isolated respiring mitochondria may be attributed, at least in part, to differences in the ability of the preparation to consume oxygen under the specific experimental conditions employed in the assay, including temperature, protein concentration and respiratory rates. Therefore, isolated mitochondria, respiring *in vitro* under conditions of forward electron transport, release ROS with Complex I- and II-linked substrates in the resting condition (state 4) and when energy demand is maximal (state 3), provided that there is sufficient oxygen dissolved in the medium.

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