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Research Paper

Oxygen-dependence of mitochondrial ROS production as detected by Amplex Red assay

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ABSTRACT

The initial rates of superoxide *plus* hydrogen peroxide (ROS) generation by intact or permeabilized rat heart mitochondria and coupled inside-out bovine heart submitochondrial particles (SMP) oxidizing NAD-dependent substrates, NADH, and succinate were measured by detecting resorufin formation in the Amplex Red assay at various oxygen concentrations. Linear dependences of the initial rates on oxygen concentration within the range of ~125–750 μM were found for all significant mitochondrial generators, i.e. the respiratory complexes and ammonium-stimulated dihydrolipoamide dehydrogenase. At lower oxygen concentrations upon its decrease from air saturation level to zero, the time-course of resorufin formation by SMP catalyzing coupled oxidation of succinate (the total ROS production by respiratory complexes II and III and by the reverse electron transfer (RET)-mediated by complex I) also corresponds to the linear dependence on oxygen with the same first-order rate constant determined in the initial rate studies. Prolonged incubation of SMP generating succinate-supported complex I-mediated ROS affected neither their NADH oxidase nor ROS generating activity. In contrast to SMP significant deviation from the first-order oxygen dependence in the time-course kinetics during coupled oxidation of succinate by intact mitochondria was evident. Complex I catalyzes the NADH:resorufin oxidoreductase reaction resulting in formation of colorless reduced resorufin. Hydrogen peroxide oxidizes reduced resorufin in the presence of peroxidase, thus showing its dihydroresorufin peroxidase activity. Combined NADH:resorufin reductase and dihydroresorufin peroxidase activities result in underestimation of the amount of hydrogen peroxide generated by mitochondria. We conclude that only initial rates of the mitochondrial ROS production, not the amount of resorufin accumulated, should be taken as the reliable measure of the mitochondrial ROS-generating activity, because of the cycling of the oxidized and reduced resorufin during Amplex Red assays fed by NADH and other possible reductant(s) present in mitochondria.

1. Introduction

Partially reduced oxygen (superoxide radical, hydrogen peroxide, hydroxyl radical) conventionally called reactive oxygen species (ROS) can result in deleterious oxidative stress if overproduced or serve as physiologically indispensable metabolites when present at their normal level. Since publication of seminal paper by Gershman et al. in 1954 [1] and discovery of hydrogen peroxide formation by antimycin-inhibited submitochondrial particles (SMP) [2], numerous reports have been published on general properties of mitochondrial ROS production, substrate donors, sites where they form, and their pathophysiological significance. Excellent accounts on those particular aspects reviewed by different research groups are available [3–10]. Less attention has been paid to a dependence of ROS production on oxygen, an obligatory participant in the process, and somehow controversial data have been reported. Linear dependence of hydrogen peroxide production on oxygen by mitochondria isolated from various tissues, species, and isolated respiratory complex I on oxygen concentration have been reported [11–16], whereas hyperbolic and substrate-donor- and respiratory state-dependent dependence have also been narrated for rat liver mitochondria [17,18]. Previously, we found first order rate, i.e. linear dependence on oxygen, of succinate-supported energy-linked superoxide production by bovine heart SMP [19] as detected by reduction of acetylated cytochrome *c* [20] upon continuous oxygen consumption starting from normal atmospheric air saturation down to about 10 μM. Physiological concentrations of oxygen in various tissues

Abbreviations: AR, Amplex Red, 10-acetyl-3,7-dihydrophenoxazine; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FMN and $FMNH_2$, flavin mononucleotide, oxidized and reduced forms, respectively; HP, horseradish peroxidase; Res and $ResH_2$, resorufin and dihydroresorufin, respectively; RET, respiratory complex I-mediated reverse electron transfer; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase

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in different metabolic states are diverse and significantly lower (5–10-fold) than that in air-saturated solutions [21,22]. Therefore, the oxygen dependence of ROS production at its low concentrations seems to be particularly relevant for cell physiology.

Accurate determination of ROS in biological samples is far from trivial because of their inherent high and unspecific reactivity and very low steady-state concentrations [23]. Several methods have been developed for reliable measurements of ROS production [24,25]. Amplex Red (AR) assay [26,27] seems among most widely used for hydrogen peroxide detection. The reactions involved in H_2O_2 quantitative detection are following:

$$\underset{\text{Colorless, non fluorescent}}{AR} + H_2O_2 \xrightarrow{\text{peroxidase}} \underset{\text{Colored, fluorescent}}{\text{resorufin(Res)}} + H_2O + \text{acetate}$$

If catalytically competent peroxidase and superoxide dismutase (SOD) are present the stoichiometry (H_2O_2 + 2 superoxide)/Res is 1. Because of high absorption/fluorescence of Res and the relative specificity of the acetyldihydrophenoxazine peroxidase reaction, very low concentration of H_2O_2 can be detected, although several possible pitfalls due to involvement of required auxiliary enzymes (peroxidase and SOD) have been discussed [28–31].

The original purpose of this study was to evaluate a dependence of heart mitochondria ROS production over a wide range of oxygen concentrations, particularly at its low concentrations. Our observations on AR assay prompted us to look closer at some properties of resorufin, which should be taken into account if used for mitochondrial ROS production.

2. Materials and methods

Rat heart mitochondria [32,33] were prepared as described. Adult rats (3–6 months of age) were treated according to "The rules of research activity in biology, medicine, and other related areas" approved by the Russian Federation acts according to the international standards (GLP). Bovine heart inside-out coupled and activated SMP [34,35] were prepared from bovine hearts obtained from slaughterhouse material in Moscow. Mitochondria and SMP were assayed in the standard reaction mixture composed of 0.25 M sucrose, 5 mM potassium phosphate, 10 mM KCl, and 0.1 mM EDTA (pH 7.5) at 30 °C. Other additions to the mixture are indicated in the figure legends. The data were analyzed with the assumption that no significant difference in the mitochondrial enzymes content, location, and their specific activities exist for *Bos taurus* and *Rattus rattus*. Where indicated, the mitochondria were permeabilized in the assay mixture by preincubation with alamethicin (40 μg/ml) and 2.5 mM $MgCl_2$ for 1 min [36]. Hydrogen peroxide formation was measured photometrically with AR (10 μM) as formation of Res ($\varepsilon_{572–600}$ = 54 mM^{-1} cm^{-1} [26]) in the standard reaction mixture supplemented with horseradish peroxidase (HP, 2 units/ml) and bovine erythrocyte SOD (6 units/ml). Total hydrogen peroxide-producing activity assayed with AR in the presence of SOD is the sum of the specific H_2O_2 generation *plus* half of the superoxide-producing activity. The hydrogen peroxide assays were calibrated by the addition of proper aliquots of H_2O_2 from freshly prepared stocks made by dilution of concentrated photometrically determined (ε_{230} = 81 M^{-1} cm^{-1}) solution. Oxygen consumption was measured amperometrically with an oxygen-sensitive membrane-coated platinum electrode. AR, SOD, and HP were added to the standard oxygen assay samples at the same concentration as added to the ROS production assays; they did not affect oxygen consumption. The initial rates of succinate-supported reverse electron transfer activity (RET) catalyzed by SMP at different oxygen concentrations was measured aerobically in the standard reaction mixture supplemented with 5 mM succinate and 1 mM NAD^+. ATP-dependent RET [34] was measured as before except for 3 mM ATP•Mg^{2+} and sodium sulfide (2 mM) (to prevent respiration) were added. Protein content was determined by the biuret procedure.

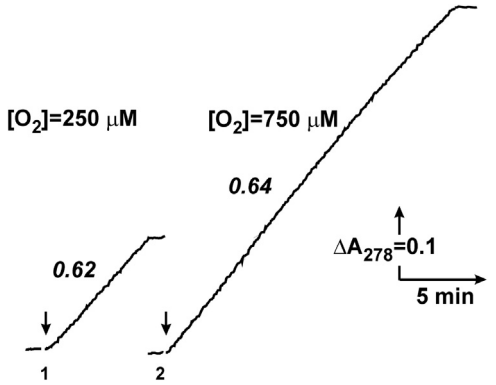

Fig. 1. An example of determination of oxygen concentration in the standard reaction mixtures used throughout this study. Uncoupled (2 μM FCCP was added) oxidation of 5 mM succinate was traced as fumarate formation determined at 278 nm ($\varepsilon\frac{mM}{278}$ = 0.3). The reaction was initiated by the addition of SMP (0.15 mg of protein per ml) (indicated by arrows) to the closed spectrophotometric cuvette with air saturated (curve 1) and standard reaction mixture saturated by pure oxygen (curve 2). Figures on the traces in italic are the specific oxidase activities (μmol/min per mg of protein). Practically no fumarate formation was seen in a reaction mixture saturated by pure argon.

Reaction mixture with various concentrations of oxygen was made by mixing of proper volumes of the solutions saturated by either atmosphere air, or argon, or pure oxygen. The actual concentration of oxygen in thus prepared mixtures was determined as follows. Succinate (5 mM) oxidation by uncoupled (2 μM FCCP) SMP was traced by following zero-order fumarate formation at 278 nm ($\varepsilon\frac{mM}{278}$ = 0.3) up to abrupt termination of the reaction, and oxygen concentration was calculated as [O_2] = 2 [fumarate]. An example of this procedure is illustrated in Fig. 1.

All data are presented as mean ± SEM of at least 3 independent experiments.

Succinate, malate, glutamate, Res, rotenone, FCCP, SOD from bovine erythrocytes (Cat. No. S7571) and from *Escherichia coli* (Cat. No S5639) were from Sigma-Aldrich (USA); NADH, HP (Cat. No. 195372) were from MP Biomedicals (USA); AR was from AnaSpec, Inc. (USA). NADH-OH was prepared essentially as described [37]. Other chemicals of highest purity available were from local suppliers.

3. Results

Fig. 2 shows the initial rates of H_2O_2 *plus* superoxide generation at various oxygen concentrations by inside-out SMP, the preparation that produces ROS by the respiratory chain components only. Linearly dependent first-order reaction with oxygen was evident for ROS generation within the 125–750 μM range of O_2. In accord with our previously published data [38], the highest rate was detected for complex I reduced by succinate via the RET as evident from strong decrease in the activity by rotenone, a specific inhibitor of the RET reaction (Fig. 2A). The addition of NADH did not affect the activity seen with succinate alone (Fig. 2B). It should be noted that we reported previously that NADH at high concentration decreases RET-induced superoxide generation [38]. The data in Fig. 2B where the rate of sum of hydrogen peroxide and superoxide production were measured under slightly different conditions revealed no inhibitory effect of added NADH. We left this apparent discrepancy for more detailed investigations in future experimentations. Complex II and the myxothiazol-insensitive site of complex III contributed less than 20% to the overall ROS production at all oxygen concentrations, although higher activity could be reached in agreement with previously reported data [35] at lower concentrations of succinate (Fig. 2C).

Linear dependence of the initial rates on oxygen concentrations