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SUPEROXIDE RADICALS AND HYDROGEN PEROXIDE FORMATION IN MITOCHONDRIA FROM NORMAL AND NEOPLASTIC TISSUES

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Summary

Mitochondria from beef heart, Morris hepatoma 3924A and Ehrlich ascites tumor (Lettré mutant) have been studied with respect to hydrogen peroxidase and superoxide radical formation and the presence of superoxide dismutase activity (EC 1.15.1.1, superoxide:superoxide oxidoreductase).

The generation of superoxide radicals and hydrogen peroxide occurs at the level of the membrane, being present also in mitochondrial fragments.

Hepatoma and ascites mitochondria have little or no superoxide dismutase activity.

Superoxide radicals appear to be precursors of hydrogen peroxide formation, the reaction being catalyzed by superoxide dismutase.

Introduction

Mitochondria from different tissues can produce hydrogen peroxide [1—4]. The generator of H_2O_2 has been localized at the level of the b-c_1 segment of the mitochondrial electron transfer chain [3] and has been shown to be dependent on the energy and reduction state of the mitochondria [3]. Coupled mitochondria have a high rate of hydrogen peroxide formation whereas uncoupled mitochondria do not produce H_2O_2 unless antimycin is present to block electron transfer [3].

It has been suggested that H_2O_2 is formed in mitochondrial membranes by a two electron reduction of molecular oxygen [4] or, alternatively, by one electron reduction followed by dismutation of the superoxide radicals formed into hydrogen peroxide and oxygen [5].

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid.

In this paper, direct measurements of superoxide radicals in mitochondrial membranes have been performed by two methods, namely the co-oxidation of epinephrine to adrenochrome [6] and the reduction of ferricytochrome *c* [7]. It has also been found that superoxide dismutase prevents epinephrine oxidation and ferricytochrome *c* reduction and, at the same time, stimulates the formation of hydrogen peroxide. In tumor mitochondria, which have been found to contain little or no superoxide dismutase activity, O_2^- radicals, but not hydrogen peroxide, are formed. It is concluded, also from the stoichiometric relationship between superoxide radicals and hydrogen peroxide, that O_2^- is a precursor of H_2O_2 formation in mitochondria.

Materials and Methods

Chemicals. L-Epinephrine, horse-radish peroxidase (EC 1.11.1.7) gradeVI, and horse heart cytochrome *c*, type VI, were obtained from Sigma Chemical Co., scopoletin (7-hydroxy-6-methoxy-cumarin) from Fluka A.G., Switzerland, xanthine oxidase (EC 1.2.3.2) from Boehringer, Mannheim; bovine erythrocuprein (superoxide dismutase, EC 1.15.1.1) was obtained from Miles Laboratories, Inc. Kankakee.

Mitochondrial preparations. Beef heart mitochondria were prepared according to Blair [8], mitochondria from Ehrlich ascites tumor, Lettré mutant, according to Kobayashi et al. [9] with slight modifications, mitochondria from Morris hepatoma 3924A according to Pedersen et al. [10].

Morris hepatoma 3924A was grown in the hind legs of female rats of the ACI/T strain and removed about 2—3 weeks after inoculation.

Mitochondrial fragments were obtained by subjecting mitochondria to sonic treatment (1 min, 3 A in a Branson Sonifier) in 2 mM EDTA, pH 9.0. Intact mitochondria were removed by low speed centrifugation (8000 × *g*, 10 min), and the remaining supernatant centrifuged for 30 min at 144 000 × *g* to precipitate membrane fragments. In some cases the supernatant was also kept for analyses.

Protein concentration was measured by a biuret method [11].

Adrenochrome method. The co-oxidation of epinephrine induced by superoxide radicals [6] was followed in a dual-wavelength spectrophotometer at 480—575 nm. The reaction mixture contained 0.25 M sucrose, 50 mM HEPES, pH 7.5, 1 mM epinephrine, 3 mM succinate and 0.5 mM EDTA. After saturation with oxygen, mitochondrial membranes were added. The reaction of radical formation was initiated by the addition of 2.5 μg/ml antimycin. The extinction coefficient of adrenochrome at the wavelength pair employed was 2.86 cm^{-1} mM^{-1}.

Cytochrome c method. Reduction of ferricytochrome *c* by superoxide radicals [7] was measured in a dual-wavelength spectrophotometer at 550—540 nm in a medium containing 0.25 M sucrose, 50 mM HEPES, pH 7.5, 5 μM rotenone, 2.5 μg/ml antimycin, 3 mM succinate and about 0.5 mg/ml mitochondrial fragments protein.

Scopoletin method. The fluorescence decrease of scopoletin (in the presence of horse-radish peroxidase) induced by hydrogen peroxide [1,12] was recorded in a Eppendorf fluorimeter, using a 366-nm interference filter for

excitation and a 470-nm cut-off filter for emission. The assay system contained: 0.25 M sucrose, 50 mM HEPES, pH 7.5, 1 μM scopoletin, 3 mM succinate, 10 μg/ml horse-radish peroxidase and 2.5 μg/ml antimycin. The last reagent initiated the reaction. For calibration, a small aliquot of hydrogen peroxide was added to the incubation medium and the fluorescence decrease obtained was used as a standard. The assay was performed under stirring in the buffer saturated with oxygen, with a stream of oxygen directed onto the surface of the solution.

Superoxide dismutase assay. The superoxide dismutase activity was assayed from the ability by different fractions of mitochondria to inhibit the oxidation of epinephrine at pH 9.0, in the presence of xanthine (1 mM), xanthine oxidase ($1.1 \cdot 10^{-8}$ M) and epinephrine (1 mM) [6].

Catalase assay. Catalase activity was measured in a Hitachi-Perkin Elmer spectrophotometer at 240 nm using the extinction coefficient 0.036 $cm^{-1} mM^{-1}$ [13].

Results

Localization of superoxide dismutase in beef heart and tumor mitochondria

Disruption of mitochondria by ultrasonic treatment results in the liberation of both the mitochondrial matrix and the content of the intermembrane space, which can be recovered in the supernatant after high-speed centrifugation of the mitochondrial membrane fragments.

The presence of superoxide dismutase can be detected in quantitative terms by the inhibition of epinephrine oxidation to adrenochrome, using as a generator of superoxide radicals xanthine plus xanthine oxidase [6].

In the experiments reported in Table I the concentrations of xanthine and xanthine oxidase were adjusted to give a constant rate of adrenochrome forma-

TABLE I

SUPEROXIDE DISMUTASE ACTIVITY IN DIFFERENT TYPES OF MITOCHONDRIA

The reaction mixture contained: 0.25 M sucrose, 50 mM HEPES, pH 9.0, 1 mM xanthine and $1.1 \cdot 10^{-8}$ M xanthine oxidase. The measurements were performed in a dual-wavelength spectrophotometer at 480—575 nm. Protein concentration in experiment 1 was 1.0 mg · ml^{-1}, for mitochondrial fragments and an amount of supernatant corresponding to 0.6 mg · ml^{-1} mitochondrial fragments. In experiment 2 protein concentration was 1.6 mg · ml^{-1}. Fragmentation was obtained in a Branson Sonifier at 3 A for 1 min. In experiment 2, the whole suspension of sonic-treated mitochondria was utilized; in experiment 1 fragments and supernatant were tested separately after separation of the fragments.

Additions	Adrenochrome (nmol · min^{-1})	Inhibition (%)
Experiment 1		
Xanthine + xanthine oxidase	12.0	—
+ mitochondrial fragments	10.0	16
+ supernatant of 0.6 mg · ml^{-1} mitochondrial fragments	6.0	50
Experiment 2		
Xanthine + xanthine oxidase	10.6	—
+ beef heart mitochondria	3.3	71
+ Ehrlich ascites mitochondria	9.8	7
+ Morris hepatoma mitochondria	11.2	0

tion of 10—12 nmol · min^{-1}. In the presence of mitochondrial fragments, a 16% inhibition in adrenochrome formation was observed with 1 mg mitochondrial membrane protein/ml. With an amount of supernatant corresponding to the soluble fraction of 0.6 mg/ml of membrane fragments, 71% inhibition of adrenochrome formation was obtained.

These results appear to confirm that mitochondria, also from beef heart, contain superoxide dismutase(s) localized in the soluble fraction [14].

The slight inhibition of adrenochrome formation produced by mitochondrial membrane fractions was not abolished by three subsequent washings of the fragments, each of them preceded by an ultrasonic treatment to facilitate the release of adsorbed or trapped enzymes.

In Table I the superoxide dismutase activity of the entire mitochondrial fraction (after ultrasonic disruption) of tumor and normal mitochondria is also shown. Ehrlich ascites mitochondria had only a slight inhibitory activity on the xanthine plus xanthine oxidase-induced adrenochrome formation, while Morris hepatoma mitochondria had no detectable activity. It can be concluded that tumor mitochondria have little or no superoxide dismutase activity as compared to mitochondria from normal tissues.

Localization of the generator of superoxide radicals in the mitochondrial membrane

The fragmentation procedure described above affords the isolation of a membrane fraction with very little superoxide dismutase activity and permits an evaluation of the rate of superoxide radicals production.

In Fig. 1 an experiment is reported where mitochondrial fragments were

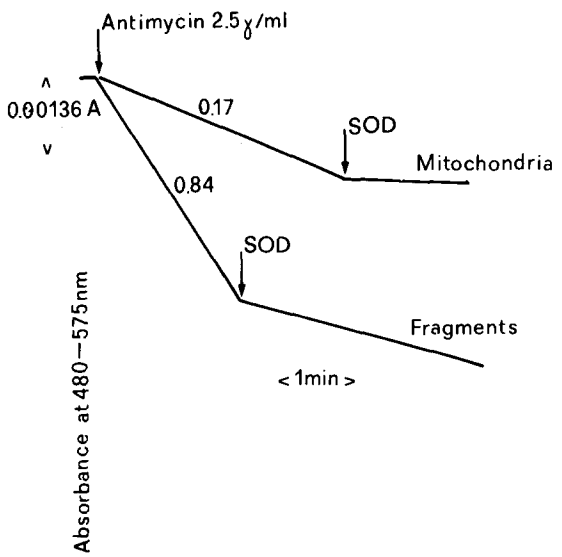

Fig. 1. Oxidation of epinephrine to adrenochrome in the presence of intact and fragmented mitochondria from beef heart. Experimental conditions: 1.2 mg protein · ml^{-1} (both in the case of intact and fragmented mitochondria) were suspended in an oxygen-saturated buffer containing 0.25 M sucrose, 50 mM HEPES, pH 7.5, 1 mM epinephrine and 3 mM succinate. The reaction was started by the addition of antimycin. Superoxide dismutase (SOD) was added at a concentration of $2 \cdot 10^{-6}$ M. The number on the traces represent nmol adrenochrome formed · min^{-1} · mg^{-1}.

TABLE II

ADRENOCHROME FORMATION IN MITOCHONDRIAL MEMBRANE FRAGMENTS

Membrane fragments (1–2 mg protein · ml^{-1}) were suspended in 0.25 M sucrose, 50 mM HEPES pH 7.5, 3 mM succinate, 0.5 mM EDTA, 1 mM epinephrine, 2 $\mu g \cdot ml^{-1}$ antimycin and 1 μM superoxide dismutase. Measurements were performed in a dual-wavelength spectrophotometer at 480–575 nm. Values are given in nmol adrenochrome formed · $min^{-1} \cdot mg^{-1}$.

Mitochondrial fragments	- Dismutase	+ Dismutase
Beef heart	0.85	0.02
Ehrlich ascites	0.90	0.02
Morris hepatoma	3.86	0.05

incubated in the presence of 1 mM epinephrine and 3 mM succinate. The addition of antimycin, which is known to induce reduction of all the components of the respiratory chain on the substrate side of cytochrome c_1, initiated the formation of adrenochrome at a rate of 0.84 nmol · min^{-1} · mg^{-1} protein. The oxidation of epinephrine under the conditions employed (0.5 mM EDTA and pH 7.5) was not consistent with an auto-oxidation but rather with a superoxide radical-mediated co-oxidation to adrenochrome. Thus, superoxide radicals appear to be produced by mitochondria and their rate of formation is higher in the isolated membranes than in intact mitochondria (0.17 nmol · min^{-1} · mg^{-1}). This suggests that the endogenous mitochondrial superoxide dismutase is effective in catalyzing dismutation of the radicals produced at the membrane level to H_2O_2 and oxygen (see below).

The residual formation of superoxide radicals in intact mitochondria and the total production in fragments was sensitive to added superoxide dismutase, a further indication that O_2^- radicals are indeed detected by the reaction of adrenochrome formation.

A comparison between the ability of membrane fragments from normal and tumor mitochondria to produce O_2^- radicals is presented in Table II. In ascites mitochondria the rate of adrenochrome formation was of the same order as that of beef heart mitochondria, but in hepatoma membrane fragments the rate was 5-times higher. In all cases the formation of adrenochrome was superoxide dismutase sensitive.

Hydrogen peroxide formation in intact and fragmented mitochondria

The production of hydrogen peroxide, as detected by the scopoletin method, was higher in intact than fragmented mitochondria (Fig. 2). As described previously, the formation of hydrogen peroxide was studied by using succinate as a substrate and antimycin to completely reduce the mitochondrial electron carriers before cytochrome c_1.

Addition of 2 μM superoxide dismutase stimulated the rate of hydrogen peroxide formation more than two-fold in mitochondrial fragments, but had no effect on the rate of hydrogen peroxide formation in intact mitochondria. When either ascites or hepatoma mitochondria were employed, no significant amounts of hydrogen peroxide could be detected in the absence of superoxide dismutase.

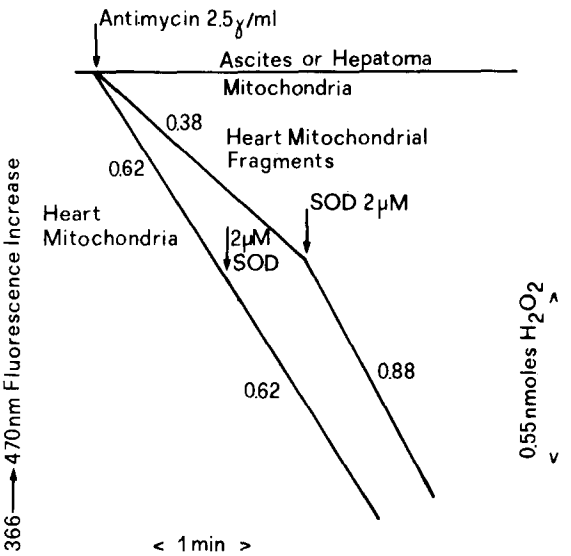

Fig. 2. Hydrogen peroxide formation in intact and fragmented mitochondria. Experimental conditions: 0.7 mg protein · ml^{-1} was used in all experiments. The medium contained: 0.25 M sucrose, 50 mM HEPES pH 7.5, saturated with oxygen, 1 μM scopoletin, 3 mM succinate, 10 μg/ml antimycin. The reaction was initiated by the addition of antimycin.

Since the detection of hydrogen peroxide may be highly affected by reactions leading to a distruction of H_2O_2, the presence of catalase activity was tested in all the preparations employed. While mitochondrial preparations from beef heart or ascites tumor cells were always free of catalase, in hepatoma mitochondria only trace amounts were found.

Relationship between superoxide radicals formation and hydrogen peroxide production in beef heart mitochondria

Reduction of cytochrome *c* by superoxide radicals is a sensitive and quantitative test for O_2^- formation. The reaction is highly sensitive to superoxide dismutase.

In order to have a quantitative relationship between the rate of superoxide radical and of hydrogen peroxide formation in beef heart mitochondrial fragments, the cytochrome *c* reduction test was employed, and in parallel experiment H_2O_2 was measured by the scopoletin test. The use of cytochrome *c* in the detection of oxygen radicals was found possible only in mitochondrial fragments, where the cytochrome *c* binding sites are not accessible to added cytochrome *c*, and consequently its enzymatic reduction and oxidation are negligible.

Mitochondrial fragments were incubated in the presence of 2.5 μg · ml^{-1} antimycin and 50 μM ferricytochrome *c* (Fig. 3). After addition of 1.5 mM succinate the rate of cytochrome *c* reduction was followed spectrophotometrically at 550 minus 540 nm. At pH 7.5 the rate of reduction was 1.5 nmol · min^{-1} · mg^{-1} and was higher at pH 8.0 and lower at pH 7.0. The reduction of cytochrome *c* obtained in the experimental conditions described above was

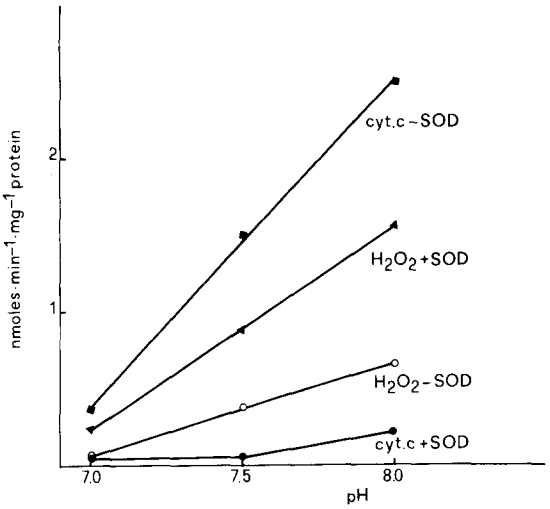

Fig. 3. Correlation between the rate of O_2^- radical formation and that of hydrogen peroxide in mitochondrial fragments. Experimental conditions: for hydrogen peroxide determination as in Fig. 2. For cytochrome *c* reduction the reaction medium was: 0.25 M sucrose, 50 mM HEPES, 50 μM cytochrome *c*, 5 μM rotenone, 2.5 $\mu g \cdot ml^{-1}$ antimycin. When superoxide dismutase (SOD) was present its concentration was 2 μM. Protein concentration (beef heart mitochondrial fragments) 0.28 $mg \cdot ml^{-1}$.

completely sensitive to superoxide dismutase, indicating that the reduction of cytochrome *c* was caused by superoxide radicals.

Measurement of hydrogen peroxide under separate conditions, in the presence of added superoxide dismutase, gave values in the same mitochondrial fragment preparation very close to one half of the amount of radicals produced. The effect of pH on H_2O_2 production was parallel to that on superoxide radical formation.

The average ratio of the rate of O_2^- produced and of H_2O_2 was close to 2 in the whole pH range considered. For example, at pH 8.0, four different mitochondrial fragment preparations gave a O_2^-/H_2O_2 ratio of 2.03 ± 0.25, and similar values were found at all pH values.

Discussion

Detection of superoxide radicals in mitochondrial membranes

The numerous techniques currently employed for the detection of superoxide radicals have severe limitations when applied to biological membranes. Mitochondria for instance can enzymatically reduce nitrobluetetrazolium and ferricytochrome *c* and the luminol light emission is not sufficiently specific for superoxide radicals.

Epinephrine co-oxidation to adrenochrome, produced by superoxide radicals, shows no interference due to the presence of mitochondrial membranes, and can therefore be safely employed for the detection of superoxide radicals generated by mitochondrial membranes.

In certain specific circumstances cytochrome *c* can also be employed in the detection of O_2^- formation; in mitochondrial fragments, in fact, oxidation

or reduction of cytochrome *c* via its reductase and oxidase are negligible, due to the inaccessibility of cytochrome *c* to its binding sites [15].

With both techniques it was shown that mitochondrial membrane fragments (which contain very little superoxide dismutase) produce measurable amounts of superoxide radicals. In the case of hepatoma mitochondria, which do not contain dismutase, the rate of radical formation is several-fold higher than in heart mitochondrial fragments.

Distribution of superoxide dismutase in different mitochondrial species

The use of the xanthine plus xanthine oxidase test to detect the presence of superoxide dismutase has permitted the determination that two mitochondrial species, from ascites tumor cells and from Morris hepatoma, contain little or no superoxide dismutase. It has been reported recently that mitochondrial superoxide dismutase is absent in SV-40-transformed embryonic cells [16], while the cytosolic type is represented normally. The finding of this study, that ascites and hepatoma mitochondria are also devoid of superoxide dismutase, may indicate that it is a general feature of tumor mitochondria not to possess superoxide dismutase.

Correlation between hydrogen peroxide and superoxide radical formation

The origin of mitochondrial hydrogen peroxide resides in the dismutation of superoxide radicals formed at the level of the electron transport chain of mitochondria. Evidence for this conclusion is afforded by the following observations:

1. Mitochondrial fragments, that contain little dismutase, form less hydrogen peroxide than intact mitochondria containing dismutase. 2. Tumor mitochondria, free of dismutase, do not form hydrogen peroxide but only superoxide radicals. 3. There is a stoichiometric relationship between superoxide radicals formed in mitochondrial fragments and the hydrogen peroxide produced in the presence of superoxide dismutase, with a ratio of 2, as expected for a dismutation reaction. 4. Mitochondria from brain cells, which do not form radicals, do not produce hydrogen peroxide despite the presence of superoxide dismutase [17].

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