OXYGEN RADICALS AND HYDROGEN PEROXIDE IN RAT BRAIN MITOCHONDRIA

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1. Introduction

Production of hydrogen peroxide in cells is a general phenomenon and, in particular, subcellular organelles such as peroxisomes [1], microsomes [1] and mitochondria [1,2] have been shown to be a source of H_2O_2. A number of tissues have been found to contain mitochondria, which are able to form H_2O_2 under some conditions both in vitro and in vivo. In particular mitochondria from liver, heart and kidney have been shown to produce relatively high amounts of H_2O_2 [3,9].

The role of H_2O_2 in cells is still a matter of discussion, and in particular that of H_2O_2 produced in mitochondria. Since the finding that heart mitochondria produce superoxide radicals and may dismutate them into H_2O_2 [4], the possible production of O_2^- by other types of mitochondria must also be evaluated. Moreover the comparison between mitochondria coming from different tissues may cast some light on the possible role of O_2^- and H_2O_2 in cells. In the present study the production of H_2O_2 in mitochondria from rat brain has been investigated. It was found that, contrary to heart and liver mitochondria, but similarly to Ehrlich ascites tumor cell mitochondria [5] they do not produce $H_2O_2 \cdot O_2^-$ radicals are produced in all mitochondrial species tested including mitochondria from ascites tumor cells, except brain mitochondria.

2. Materials and methods

The reagents and enzymes used in our study were purchased from the following: Scopoletin (7-hydroxy-6-methoxy-cumarin) from Fluka A. G., Switzerland; L-epinephrine (adrenaline) from Sigma Chemical Co.; luminol (5-amino-2,3-dihydro-1,4-phalazinedione) from Eastman Kodak Co., Rochester, N.Y., USA); horse radish peroxidase (EC 1.11.1.7) (HRP) grade VI, from Sigma Chemical Co., catalase (EC 1.11.1.6) from Boehringer, Mannheim.

Pure superoxide dismutase ($\epsilon_{280}/\epsilon_{259} = 0.59$) isolated from bovine blood (bovine erythrocuprein) was a gift of Dr. U. Weser, University of Tübingen.

2.1. *Determination of H_2O_2*

Mitochondrial H_2O_2 formation was measured by the scopoletin method in an Eppendorf fluorometer the following way: 1.5 mg/ml of mitochondrial protein [6] (respiratory control generally around 3–4 with succinate) were supplemented with 2 μM scopoletin and 0.5 μM HRP in sucrose–Tris buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4), saturated with oxygen. The scopoletin fluorescence changes were measured with the Eppendorf 366 Hg as primary and the 380–3000 nm secondary filter. The reduced scopoletin reacts with H_2O_2 via HRP with one to one stoichiometry and loss of its fluorescence. The rate of scopoletin fluorescence decrease is therefore

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directly proportional to the rate of hydrogen peroxide formation.

2.2. *Determination of oxygen radicals*

2.2.1. Epinephrine oxidation test

About 1 mg protein/ml of membrane fragments obtained by sonication of mitochondria were supplemented with 1 mM epinephrine, 0.5 μM catalase and 3 mM succinate in 0.25 M sucrose and 50 mM HEPES buffer, pH 8.6. The rate of adrenochrome formation, which was observed after the addition of 0.5 μg antimycin/mg protein, was measured at 480–575 nm with a dual wavelength spectrophotometer using the difference of extinction coefficients of 2860 M^{-1} cm^{-1}.

2.2.2. Luminol chemiluminescence

0.5 mg protein/ml of SMP in the same medium, were supplemented with 3 mM succinate and 1 mM luminol. Light emission observed upon addition of antimycin (0.5 μg/mg protein) was followed in the tritium channel of a Beckman scintillation counter, model CPM-100, and printed out every 20 sec as counts per minute.

2.3. *Determination of catalase activity*

Catalase activity was measured at 240 nm in a Hitachi–Perkin–Elmer spectrophotometer (Mod. 124) in 0.25 M sucrose–10 mM Tris buffer, pH 7.4. In the two cuvettes of the spectrophotometer, 0.5 mg of mitochondria were added. Subsequently H_2O_2 was added to the sample cuvette to obtain an absorbance of about 1.3 and the decrease of absorbance recorded as a function of time.

2.4. *Preparation of mitochondria*

Rat brain mitochondria were prepared by the following procedure: Wistar Albino rats were killed by decapitation. The brains were quickly withdrawn and the grey matter separated and immersed in cold sucrose (0.25 M) containing EDTA (2 mM, pH 6.8). The brain was cut in pieces and gently homogenized with a Potter homogenizer. The homogenized tissue was transferred to Sorvall centrifuge tubes and spun at 5000 rpm for 1′ in R C-2 B Sorvall refrigerated centrifuge. The supernatant was withdrawn and recentrifuged at 8000 rpm for 10′ followed by a centrifugation at 12 000 rpm for 10′.

Light material was separated from the pellet by adding a small amount of buffer which was subsequently discarded. The pellet was resuspended in the buffer and centrifuged at 8000 rpm for 7′ and subsequently at 15 000 rpm for 8′. The pellet was washed again with a small amount of buffer and resuspended in few drops of buffer. Protein concentration was measured by the biuret method [7]. The average respiratory control index was between 2–4 with succinate as substrate. The purity of the preparation was checked by measuring the lactic dehydrogenase activity which was similar to values reported in the literature [8].

3. Results and discussion

3.1. *Hydrogen peroxide production in rat brain mitochondria*

The rate of formation of H_2O_2 was measured by the scopoletin technique (fig. 1). Rat brain mitochondria (1.5 mg/ml) were incubated in 0.25 M sucrose–10 mM Tris–HCl, pH 7.4, saturated with oxygen in an Eppendorf fluorometer. Scopoletin (2 μM) gave rise to a fluorescence increase. Subsequently succinate and horse radish peroxidase were added without an appreciable decrease in fluorescence. Block of the respiratory chain by antimycin in partly coupled mitochondria stimulated an increase in the rate of H_2O_2 formation [9]. Antimycin however was ineffective in brain mitochondria. On the other hand when an equal amount of beef heart mitochondria was present, a decrease of fluorescence with time was observed, corresponding to a rate of H_2O_2 production of 0.8 nmoles/min. An apparent lack of H_2O_2 formation by brain mitochondria can be the result of lack of production or a high rate of H_2O_2 disruption. Consequently the catalase activity of isolated brain mitochondria was tested.

3.2. *Catalase activity of rat brain mitochondria*

The catalase activity of rat brain mitochondria was measured as described in Materials and methods. When rat brain mitochondria were used, no decrease in H_2O_2 absorbancy at 240 nm was recorded [10], but the addition of catalase brought rapidly the absorbance to low values indicating H_2O_2 disruption. When, instead, rat liver mitochondria were present

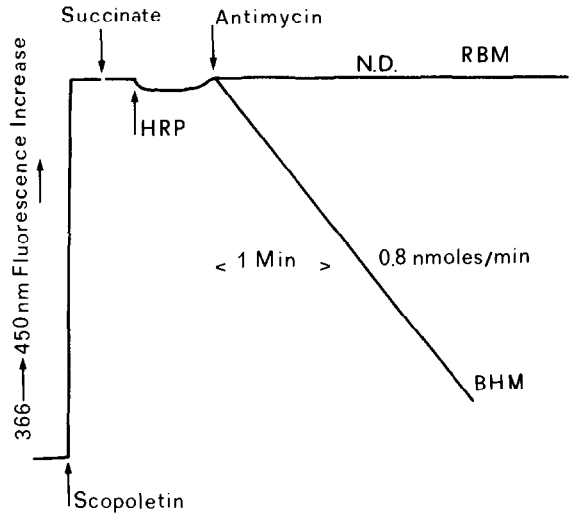

Fig. 1. Hydrogen peroxide production in rat brain and beef heart mitochondria. Experimental conditions: the production of H_2O_2 was measured by the Scopoletin method [6] in an Eppendorf fluorimeter (excitation 366 nm emission 380–3000 Eppendorf filters) in the following oxygen saturated medium: 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, and 1.5 mg/ml rat brain mitochondria. Addition of scopoletin (2 μM) produced an increase in fluorescence. 0.5 μM horse radish peroxidase was subsequently added to measure H_2O_2 formation coupled to the fluorescence decrease of scopoletin. Antimycin (2 μM) added after 2 mM succinate stimulated the production of H_2O_2 in heart but not in brain mitochondria. RBM = rat brain mitochondria; BHM = beef heart mitochondria. N.D. = not detectable.

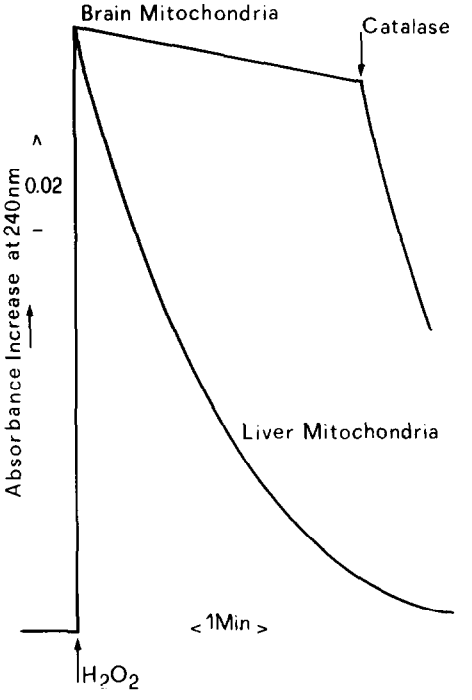

Fig. 2. Catalase activity in rat brain and rat liver mitochondria. Catalase activity was measured in a double beam spectrophotometer (Perkin–Elmer, mod. 124) at 240 nm. The two cuvettes of the spectrophotometer contained 0.5 mg of rat brain mitochondria or rat liver mitochondria in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4. H_2O_2 was subsequently added to the sample cuvette and the absorbance increase recorded. The decrease of absorbance is a consequence of the disapperance of H_2O_2. Catalase was 0.5 μM.

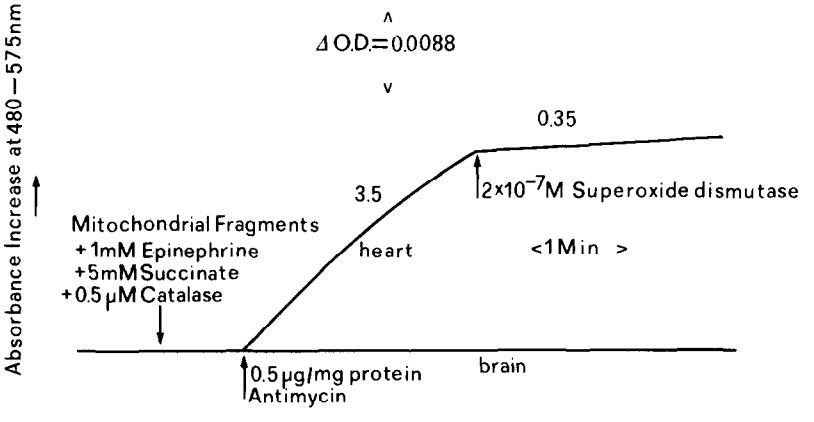

Fig. 3. Antimycin induced oxidation of epinephrine in the presence of mitochondrial fragments. Experimental conditions are indicated in Materials and methods. Protein concentration was 1 mg/ml. The numbers in the figure are nmoles/min/mg protein of adrenochrome formed.

a rapid decrease of absorbance was seen (fig. 2).

This experiment indicates that catalase activity in rat brain mitochondria is very low, not more than 1/20th of that in rat liver mitochondria and cannot reasonably account for the lack of H_2O_2 detection.

3.3. *Oxygen radicals formation in brain mitochondria fragments*

The adrenochrome test was employed to detect the formation of O_2^- radicals in membrane fragments. Because of the presence of dismutase in intact mitochondria, if radicals were formed, they would rapidly dismutate to H_2O_2 and O_2. Fragments are membranes washed free of contaminating enzymes, affording thus an ideal material to test the possible formation of radicals.

Sonicated membrane fragments from brain mitochondria were tested (fig. 3) in a dual wavelength spectrophotometer using 480–575 nm, in the presence of 1 mM epinephrine, to detect the formation of adrenochrome. No oxidation of epinephrine was detected in rat brain mitochondria fragments supplemented with succinate and antimycin while in heart fragments about 3.5 nmoles of adrenochrome/min/mg protein were formed. Addition of superoxide dismutase (2×10^{-7} M) produced an almost complete inhibition of adrenochrome formation, indicating that the oxidation of epinephrine is caused by O_2^- radicals [4]. Thus apparently no detectable oxygen radicals are produced in rat brain mitochondria. A similar conclusion was reached by using the chemiluminescence of luminol to detect the formation of oxygen radicals.

4. Conclusions

Rat brain mitochondria, at variance from mitochondria from other sources, do not produce oxygen radicals nor hydrogen peroxide. Moreover they do not appear to have detectable catalase activity. Thus the presence of these reactions in mitochondria from tissues other than brain, may not be an accident or an artifact of the preparation. Moreover while only speculations are possible at the present time, the absence of O_2^- and H_2O_2 production in mitochondria may be of importance in the function of the brain cell.

Acknowledgements

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