The effect of superoxide generation on the ability of mitochondria to take up and retain Ca^{2+}

E.J. Harris†, R. Booth+ and M.B. Cooper*

Department of Biophysics, University College London, Gower Street, London WCIE 6BT, England

Received 3 August 1982

When heart or liver mitochondria are exposed to superoxide radicals generated from xanthine + xanthine oxidase their ability to take up and to retain Ca^{2+} is impaired. The rate of oxidation of pyruvate + malate as substrates is diminished and the appearance of thiol groups when the mitochondria are supplied with these substrates is abolished. These inhibitory effects are offset if respiration is supported by succinate in presence of rotenone provided that a substrate (β-hydroxybutyrate) is provided to maintain the reduction of NADH. The data agree with the thesis that a generation of thiol groups is essential to maintain membrane integrity and that the generation depends on provision of reduced NAD(P)H.

Mitochondrial Ca^{2+} *Superoxide effect* *Thiol groups*

1. INTRODUCTION

In the sequence of pathological changes following myocardial ischaemia, cellular [Ca^{2+}] increases have been described [1]. Upon subsequent reoxygenation the mitochondria are seen by electron microscopy to have undergone ultrastructural damage [2]. Mitochondria prepared from such tissue have low respiratory rates and poor control of respiration by ADP [3]. Much clinical interest has been focussed on the mechanism by which the damage occurs. Evidence from studies with isolated mitochondria has shown that an excessive loading of Ca^{2+}, such as may be expected on energising mitochondria in a high Ca^{2+} environment, leads to a high efflux of the ion giving rise to stimulation of the endogenous phospholipase and consequent high permeability of the membrane. The lipase activity generates both fatty acids [4] and lysophospholipids [5–8] which themselves are known to cause mitochondrial swelling and loss of specific permeability. This proposed sequence of events does not take into account the possibility that active oxygen radicals, such as superoxide, O_2^- [9], may be generated from the reduced components [1] of the mitochondrial electron-transport chain when the oxygen supply is restored. Although mitochondrial superoxide is known to be removed by superoxide dismutase a rapid superoxide generation particularly if catalytic amounts of Fe^{2+} are present, can lead to formation of peroxides from the unsaturated fatty acids [11–13]. It has been suggested that lipid peroxidation may occur during reoxygenation of the ischaemic heart [13] and the beneficial action of antioxidants such as α-tocopherol [14] and ubiquinone [15] have been demonstrated in the perfused rabbit heart undergoing a cycle of ischaemia and reoxygenation. In this latter work the performance of mitochondria isolated from the tissue was used to assess damage. Certain cardiac conditions may be correlated with ubiquinone deficiency though the mode of action has not been explained [16].

Here, we present evidence for a mechanism of mitochondrial damage associated with the generation from an exogenous source of superoxide radicals. The consequence is to deplete the thiol content and to render the mitochondria less resistant to the consequence of uptake of Ca^{2+} and its associated Ca^{2+} efflux.

Present addresses: †Department of Biophysical Chemistry, Royal Free Hospital, Hunter St, London WC1; +Searle Research and Development, High Wycombe, Bucks; *Chester-Beatty Research Inst., Cranbrooke, Kent, England

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

2. METHODS

Mitochondria were prepared from rat liver [17] and rat heart [18]. The superoxide generating system used was a mixture of xanthine (0.5 mM) and xanthine oxidase (22 or 44 mU). This mixture was applied to a suspension of ~1.5–2.5 mg mitochondrial protein in 150 μl medium (150 mM KCl, 20 mM Tris–Hepes (pH 7.4) for a timed period after which was added 2.5 ml of a medium having 300 mM mannitol, 20 mM Tris–Hepes (pH 7.4), 1.2 mM Tris–malate and Tris–pyruvate, 0.6 mM Tris–phosphate, 0.4 mg bovine serum albumin/ml and 100 μM arsenazo III as Ca^{2+} indicator. The responses of the indicator to changes of external [Ca^{2+}] were followed by recording the differential absorbance changes ($A_{685} - A_{665}$) in an Aminco DW2 spectrophotometer. Most experiments were made by first applying a Ca^{2+} load of 50 or 100 nmol to the mitochondrial suspension in the cuvette. If uptake became complete an addition of ruthenium red (0.8 μM) was made to inhibit inward flux so that the efflux could be observed; the system was calibrated by additions of $CaCl_2$.

The production of thiol groups by the mitochondria was monitored using the reduction of dithiobis (4-nitrobenzoic) acid (100 μM) as indicator in the cuvette with the wavelength pair 520–412 nm. The sensitivity was calibrated by known additions of cysteine in solution. Exposure to the superoxide generator was in the cuvette in 2.5 ml by adding xanthine to 0.12 mM and 60 mU xanthine oxidase; the effect was immediate.

Respiratory rates were measured in a medium having 135 mM KCl, 20 mM Tris–Hepes (pH 7.2), 1 mM Tris–P_i, and 2 mg bovine serum albumin/ml. Prior exposure of the mitochondria to the superoxide-generating mixture was carried out on 1.5–2.5 mg mitochondrial protein added to 0.15 ml of the KCl medium having 0.3 mM xanthine and 11 mU xanthine oxidase.

3. RESULTS

Heart and liver mitochondria rapidly and completely take up Ca^{2+} over 35–70 nmol/mg protein. After addition of ruthenium red the rate of Ca^{2+} efflux is low (fig.1, table 1). When such a preparation had been exposed to the superoxide generating system (with 44 mU xanthine oxidase)

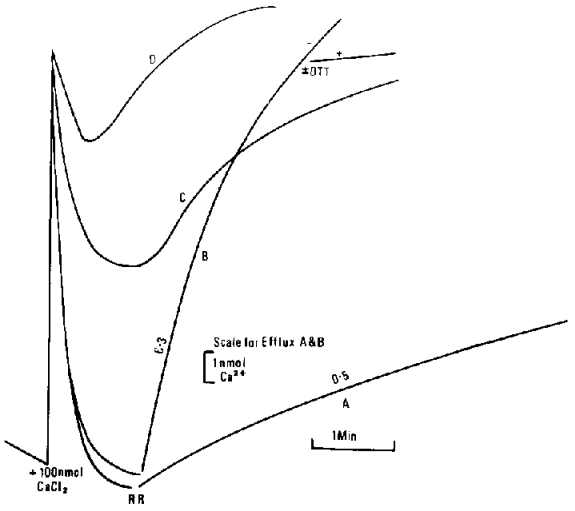


Fig.1. The effect of time of exposure of rat heart mitochondria to the superoxide generator: uptake of 100 nmol Ca^{2+} by 2 mg unexposed mitochondria; the uptake is followed by an addition of ruthenium red so that the efflux can be observed. The sensitivity has been increased for this part of the trace, the number appended is the efflux in nmol · min^{-1} · mg $protein^{-1}$; (B) record obtained from mitochondria exposed to the superoxide generator for 30 s before applying the Ca^{2+} load, after ruthenium red the efflux is now seen to be higher. At the end of curve B the consequence of adding in a parallel experiment dithiothreitol (100 μM) is illustrated; (C,D) incomplete and spontaneously reversed uptakes obtained if the mitochondria were first exposed, for 2 and 5 min respectively, to the superoxide generator; 25°C; Tris–pyruvate and –malate at 1.2 mM were present as respiratory substrates; xanthine oxidase, 44 mU.

for 30 s (fig.1B) the uptake was similar but the efflux was higher. As the time of exposure to the superoxide generator was increased there was a progressive deterioration so that, for example, the uptake process spontaneously ceased before it was completed and accumulated Ca^{2+} in the mitochondria was returned to the medium (fig.1C,D). If the pretreatment was given at 0°C instead of at 20°C so that the oxidase activity was diminished, then less effect was found on Ca^{2+} uptake and retention. Antioxidants such as reduced ubiquinone (250 μM) or butylated hydroxytoluene (25 μM) also lessened the effect while use of a mannitol medium during exposure to the superoxide pre-

Table 1

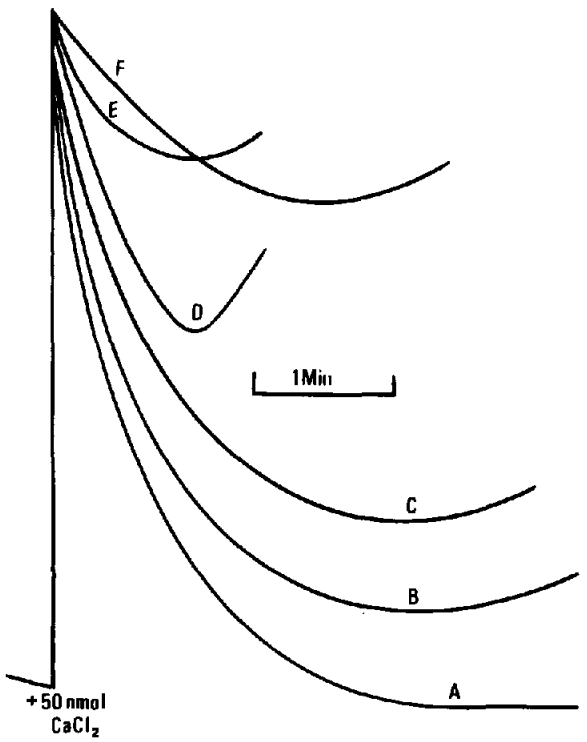
Effects of exposure to the superoxide generator on Ca^{2+} efflux from liver and heart mitochondria ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$)

Heart mitochondria						Liver mitochondria					
Untreated		After 1 min exposure		After 3 min exposure		Untreated		After 1 min exposure		After 3 min exposure	
−	+	−	+	−	+	−	+	−	+	−	+
0.5	4.3	0.6	5.3	1.75	5.5	0.55	1.1	1.1	1.25	Load not retained	
0.8	6.4	0.8	4.0	1.6	4.0	0.5	1.25	1.4	1.75	1.45	1.75
0.3	8.0	0.3	8.2	0.5	7.3	0.4	0.9	0.5	0.75	0.35	0.7
0.8	6.0	1.5	5.8	1.7	4.6	0.7	2.0	0.8	1.8	0.9	1.8
0.1[a]	4.2	0.2	6.8	0.3	7.5	0.3	0.6	0.4	0.8	0.45	1.05

[a] Mitochondria from rat injected with diethyl maleate 30 min before taking the organs for mitochondrial preparations

The mitochondria were first loaded with 30 nmol Ca^{2+}/mg, the efflux was measured in presence of 0.8 μM ruthenium red before (−) and after (+) adding NaCl to 10 mM, at 27°C; xanthine oxidase 22 mU

vented deterioration. Addition of superoxide dismutase (14 U) to the incubation, or use of heat-denatured xanthine oxidase also lessened or removed the effect.



Examples of values for Ca^{2+} efflux found after different times of exposure of either liver or heart mitochondria to the superoxide generator are set out in table 1. It appears to be the basal efflux which is increased by the exposure so that the effect does not require the presence of Na^+. In some examples, the total efflux after adding Na^+ is seen to be less from the treated mitochondria than from the controls despite their higher basal efflux. This implies that the Na^+-stimulated component diminishes, which may be ascribed to an impairment of energy supply by the superoxide

Fig.2. The effects of use of different respiratory substrates on the Ca^{2+} uptake and retention by rat heart mitochondria after exposure to the superoxide generator (B–F): (A) uptake by an untreated control. After 2 min exposure to the superoxide generator the application of Ca^{2+} (40 nmol/mg protein) in presence of 1.2 mM each of pyruvate + malate, β-hydroxy butyrate, succinate, 2.4 mM ascorbate, 40 μM tetramethylphenylene diamine led to the transient uptake in (B). The same mixture of substrates supplemented with antimycin (1 μg/ml) led to the record shown in (C); (D) succinate 1.2 mM only, in presence of rotenone; (E) pyruvate + malate, each at 1.2 mM; (F) ascorbate (2.4 mM) with tetramethylphenylene diamine (40 μM) in presence of antimycin (1 μg/ml); 25°C; xanthine oxidase, 44 mU.

treatment. The Na^+ stimulation of efflux is known to be energy-dependent [20,21].

A trial was made of the consequence of a prior (30 min) injection of diethyl maleate (0.15 g/200 g animal) because this treatment diminishes the glutathione content of the tissue [22]. The mitochondria from the injected rats appeared in this test to be more resistant to superoxide than the controls (see last line table 1). It may be that removal of tissue glutathione has stimulated its production by the mitochondria; evidently, more work on this would be of interest.

When a given preparation was tested after exposure to the superoxide generator the ability to take up Ca^{2+} was improved by adding substrates linked to sites 2 and 3 in the respiratory chain in addition to site 1 substrate. This effect is illustrated in fig.2 in which superoxide-treated mitochondria with different substrates are compared to a control (curve A). With substrates active at all sites (curve B) and no respiratory inhibitor, the uptake of Ca^{2+} is most nearly complete. When antimycin is present to restrict energy generation to site 3 but with mixed substrates to ensure that the carriers at sites 1 and 2 are kept reduced there is a considerable though transient uptake (curve C).

We examined the consequence of exposure to the superoxide generator on the ADP-stimulated respiratory rates using various substrates. After exposure of 1 min the rate in presence of pyruvate + malate at 1.2 mM each was diminished to 10% of the control; with succinate (1.2 mM) in presence of rotenone the rate fell to 60% of the control and with tetramethylphenylene diamine (40 μM) + ascorbate (2.4 mM) the rate fell to 73% of the control. The considerable inhibition seen with pyruvate + malate could explain the progressive failure of Ca^{2+} uptake seen in fig.1 as the time of exposure to the superoxide generator is increased.

It has been proposed that in the mitochondria there operates a chain of reactions starting from NADH+ATP to maintain NADP reduced by transhydrogenase activity, followed by use of the NADPH to reduce glutathione by glutathione reductase activity [23,24]. The production of thiol groups has been measured and effects of agents on the rate described [19].

In the light of experiments such as that illustrated at the top of curve B in fig.1. showing a return to a low rate of Ca^{2+} release in response to addition of dithiothreitol it seemed likely that the superoxide was acting analogously to heavy metals as a sink for thiol groups. Measurement of the thiol group production showed that when superoxide was generated the effect depended upon the sites of reaction of the substrates and respiratory inhibitors in the system. Table 2 shows complete inhibition of thiol appearance with pyruvate + malate as sole substrates in contrast to no inhibition when substrate (β-hydroxybutyrate) was provided to keep NAD reduced with electron flow blocked either between sites 1 and 2 with rotenone of between sites 2 and 3 with antimycin. Without

Table 2

Effect of exposure of mitochondria to the superoxide generator in presence of various substrates

Substrates	Rates of thiol production (nmol . min^{-1} . mg $protein^{-1}$)	
	Before exposure	After exposure
Pyruvate, 1.2 mM malate, 1.2 mM	6.2	0
β-hydroxybutyrate, 1.2 mM succinate, 1.2 mM rotenone, 0.5 μM	5.3	5.3
β-hydroxybutyrate, 1.2 mM succinate, 1.2 mM ascorbate, 2.4 mM tetramethylphenylene diamine, 40 μM antimycin, 1 μg/ml	11.3	11.8
Succinate, 1.2 mM rotenone, 0.5 μM	9.2	2.4
Ascorbate, 2.4 mM tetramethylphenylene diamine, 40 μM antimycin, 1 μg/ml	9.7	5.7

Mitochondrial protein (~2.5 mg) was used in 2.5 ml buffered KCl (135 mM) medium xanthine was added at 0.12 mM and xanthine oxidase at 60 mU

the hydroxybutyrate there is considerable inhibition in either condition.

4. DISCUSSION

There are many observations that agents which react with thiol groups impair the ability of mitochondria to take up Ca^{2+} and also accelerate the efflux of previously accumulated Ca^{2+}. Examples are mercurials [18,19], a quinone [19], diamide [5,25] and *t*-butyl hydroperoxide [24,26]. In the latter context, there is evidence [24] that it is only when glutathione peroxidase is active, so that GSH was consumed, that there is an induction of Ca^{2+} release by the peroxide.

There is general agreement that in some crucial way thiols are concerned in the maintenance or repair of the membrane in the face of Ca^{2+}-initiated damage. Additionally there is accumulating evidence that the damage associated with Ca^{2+} as measured by a general loss of internal components is due to a change to a generally permeable condition [5–8,27–29] apparently involving the activation of endogenous phospholipase by emergent Ca^{2+}.

Circumstances which give rise to free radicals, such as application of oxygen to anaerobic tissue [30] or provision of specific substrates such as alloxan in presence of glutathione and Fe^{2+} [31] are likely to render the mitochondria of the system more vulnerable to damage by Ca^{2+} loading than the normal.

ACKNOWLEDGEMENTS

We are indebted to Dr M.C.W. Evans of the Botany Department, University College of London, for the use of his DW 2 spectrophotometer. E.J.H. holds a Grant from the British Heart Foundation.

REFERENCES

[1] Shen, A.C. and Jennings, R.B. (1972) Am. J. Pathol. 67, 441–452.
[2] Hearse, D.J., Humphrey, S.M., Nayler, W.G., Slade, A. and Border, D. (1975) J. Mol. Cell. Cardiol. 8, 205–215.
[3] Lochner, A., Opie, L.H., Owen, P., Kotze, J.C.N., Bruyneel, K. and Gevers, W. (1975) J. Mol. Cell. Cardiol. 7, 203–217.
[4] Pfeiffer, D.R., Schmid, P.C., Beatrice, M.C. and Schmid, H.H.O. (1979) J. Biol. Chem. 254, 11485–11494.
[5] Palmer, J.W. and Pfeiffer, D.R. (1981) J. Biol. Chem. 256, 6742–6750.
[6] Beatrice, M.C., Palmer, J.W. and Pfeiffer, D.R. (1980) J. Biol. Chem. 255, 8663–8671.
[7] Siliprandi, F., Rugolo, M., Zoccarato, F., Toninello, A. and Siliprandi, N. (1979) Biochem. Biophys. Res. Commun. 88, 388–394.
[8] Palmer, J.W., Schmid, P.C., Pfeiffer, D.R. and Schmid, H.H.O. (1981) Arch. Biochem. Biophys. 211, 674–682.
[9] Boveris, A. and Cadenas, E. (1975) FEBS Lett. 54, 311–314.
[10] Loschen, G., Azzi, A., Richter, C. and Flohé, L. (1974) FEBS Lett. 42, 68–72.
[11] Tyler, D.D. (1975) FEBS. Lett. 51, 180–183.
[12] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.
[13] Guarnieri, C., Flamigni, F. and Caldarera, C.M. (1980) J. Mol. Cell. Cardiol. 12, 797–808.
[14] Guarnieri, C., Ferrari, R., Visioli, O., Caldarera, C.M. and Nayler, W.G. (1978) J. Mol. Cell. Cardiol. 10, 893–906.
[15] Ohhara, H., Kanaide, H., Yoshimura, R., Okada M. and Nakamura, M. (1981) J. Mol. Cell. Cardiol. 13, 65–74.
[16] Folders, K., Baker, L., Richardson, P.C., Shizukuishi, S., Kaji, M., Combs, A., Choe, J., Kwen, Y.Z., Nishii, S., Lowell, J., Lewandowski, J., Liu, F. and Hurst, G. (1980) in: Biomedical and Clinical Aspects of Coenzyme Q (Yamamura, Y. et al. eds) vol. 2, pp. 447–454, Elsevier Biomedical, Amsterdam, New York.
[17] Harris, E.J., Tate, C., Bangham, J.A. and Manger, J.R. (1971) J. Bioenerg. 2, 221–232.
[18] Harris, E.J., Al-Shaikaly, M. and Baum, H. (1979) Biochem. J. 182, 455–464.
[19] Harris, E.J. and Baum, H. (1980) Biochem. J. 186, 725–732.
[20] Crompton, M., Kunzi, M. and Carafoli, E. (1977) Eur. J. Biochem. 79, 549–558.
[21] Heffron, J.J.A. and Harris, E.J. (1981) Biochem. J. 194, 925–929.
[22] Smith, H.T., Loveridge, N., Wills, E.D. and Chayen, J. (1979) Biochem. J. 182, 103–108.
[23] Harris, E.J. (1979) Trends Biochem. Soc. 7, 770–775.
[24] Lotscher, H., Winterhalter, K., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340–4344.

[25] Siliprandi, D., Topinello, A., Zoccarato, F., Rugolo, M. and Siliprandi, N. (1975) Biochem. Biophys. Res. Commun. 66, 956–961.
[26] Lotscher, H., Winterhalter, K., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330.
[27] Harris, E.J. and Cooper, M.B. (1981) Biochem. Biophys. Res. Commun. 103, 788–796.
[28] Harris, E.J. and Chen, M.-S. (1982) Biochem. Biophys. Res. Commun. 104, 1264–1270.
[29] Rugolo, M., Siliprandi, D., Siliprandi, N. and Toninello, A. (1981) Biochem. J. 200, 481–486.
[30] Cadenas, E., Varsavsky, A., Boveris, A. and Chance, B. (1981) Biochem. J. 198, 645–654.
[31] Grankvist, K. (1981) Biochem. J. 200, 685–690.