Biochimica et Biophysica Acta, 934 (1988) 80–86
Elsevier

BBA 42773

Injury of mitochondrial respiration and membrane potential during iron / ascorbate-induced peroxidation *

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(Received 2 September 1987)
(Revised manuscript received 30 December 1987)

Key words: Lipid peroxidation; Malondialdehyde; Respiration; Membrane potential; (Rat liver mitochondria)

First functional events during peroxidation in mitochondria consisted in a progressive inhibition of the phosphorylating and uncoupled respiration with succinate and glutamate / malate as substrates, whereas the resting state respiration during the same period was virtually not influenced. The membrane potential registered at a time with the respiration rates was capable of being built up for a relatively long time interval with only minor decreases, and broke down rather promptly when the active respiration was highly diminished. Inhibition of respiration proceeded mainly during the initiation phase of peroxidation. Lag phases of varied length, of malondialdehyde formation which were predominantly attributed to the iron / protein ratios correlated closely with different time intervals needed to attain maximal inhibition of respiration and decrease in glutathione. Hence, the lessening of respiration, drop of membrane potential and loss of the antioxidant, glutathione, represent early stages in the causal chain of events which precede the onset of intensive lipid peroxidation.

Introduction

Fe^{2+}/ascorbate is a prooxidant tool commonly used to induce lipid peroxidation in various systems including mitochondria [1]. It is well known that this radical-generating process disturbs mitochondrial structure and functions. Lipid peroxidation as a complex system of reactions, involving free radical intermediates, causes oxidative degradation of unsaturated fatty acids and accumulation of a variety of peroxidized products in the membrane. Effects of peroxidative attack on energy-linked functions (respiration, membrane potential, ion transport) and membrane-associated enzymes under different experimental conditions were reported [2–6]. Furthermore, mitochondria have been shown to be protected against oxidative stress, by powerful defence systems including the activities of superoxide dismutase, catalase and glutathione peroxidase, while lipid peroxidation has been found to be strongly inhibited by various non-enzymic antioxidants [7–9]. Development of radical-induced lipid degradation is associated with breakdown of the preventive mechanisms during the exponential phase of extensive peroxidation, finally resulting in irreversible damage to mitochondria. Little is known, however, about the mechanism and the causal chain of events following radical-mediated perturbations. Thus, in order to gather further information on essential factors involved in peroxidative destruction of

* This paper is dedicated to Prof. S.M. Rapoport on the occasion of his 75th birthday.

Abbreviations: $\Delta\psi$, mitochondrial transmembrane electrical potential; DDA^+, dibenzyldimethylammonia cation; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; GSH, glutathione.

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mitochondria, it is important that functional alterations which may be reversible be detected and studied at a very early stage of the lipid peroxidation process.

It was the aim of the present work to characterize changes in mitochondrial functions in freshly isolated intact rat liver mitochondria in which peroxidation had been induced by Fe^{2+}/ascorbate. Respiration rates under various conditions, respiratory control ratios and the membrane potential were used as sensitive tools to evaluate the functional capability of mitochondria. Early effects of lipid peroxidation on the functional parameters and the glutathione content were studied in relation to each other as well as to the degree of peroxidation determined from the amount of malondialdehyde.

Materials and Methods

Isolation of mitochondria. Rat liver mitochondria were prepared from fasted animals by a standard procedure [10], washed and resuspended in 250 mM sucrose adjusted to pH 7.4 with Tris at a concentration of about 50 mg mitochondrial protein per millilitre. The functional integrity was determined by measuring the rate of oxygen uptake by a closed Clark-type electrode with ADP, succinate being used as substrate. Only mitochondria showing respiratory control ratios greater than four were used.

Iron/ascorbate-induced peroxidation. Peroxidation was initiated in a medium containing 100 mM KCl, 10 mM Tris buffer (pH 7.7) and about 5 mg mitochondrial protein/ml, by 20–40 μM ferrous sulphate and 500 μM ascorbic acid at 25 °C in an open air atmosphere. At timed intervals, samples were taken for the immediate registration of oxygen consumption and membrane potential as well as for the assay of glutathione and malondialdehyde.

Incubation conditions. The incubation medium used to assay the respiratory rate contained 100 mM KCl, 50 mM Tris (pH 7.4), 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, and 10 mM succinate or 5 mM glutamate plus 1 mM malate as respiratory substrates. In experiments adapted to measure simultaneously the respiratory parameters and the membrane potential, potassium ions in the medium were replaced by sodium ions which latter do not interact with tetraphenylboron. Essentially, the respiratory rates obtained with both media are comparable.

Membrane potential. The transmembrane potential ($\Delta\psi$) was determined from the distribution of DDA^+ between the mitochondrial matrix and the incubation medium with the aid of a DDA^+-sensitive electrode described by Muratsugu et al. [11]. The electromotive force between the DDA^+ electrode and the calomel reference electrode was measured by an electrometer (VEB Startron, Fürstenwalde, G.D.R.). The electrode was calibrated to measure the electromotive force as a function of the DDA^+ concentration in the medium. Tetraphenylboron (5 μM) was added to the reaction mixture containing 50 μM DDA^+ to facilitate permeation of DDA^+ through the mitochondrial inner membrane. The free extramitochondrial DDA^+ concentration was approximated while assuming a complex-like interaction between DDA ions and anions in the reaction mixture (succinate, inorganic phosphate). The corrected values were used to calculate the membrane potential as described in Ref. 12. Values for membrane potentials assayed by this approach with the DDA^+ electrode were consistent with data obtained by measuring the $^{86}Rb^+$ distribution [12].

Assays. Malondialdehyde formation was measured by the thiobarbituric acid method [13] which was slightly improved. The coloured complex was extracted by pyridine/butanol (1 : 15) and measured at 532 nm.

Glutathione was determined as the difference between total GSH equivalents measured by the kinetic assay according to Tietze [14], and its oxidized form (GSSG) assayed as described in [15].

Mitochondrial protein was determined by a modified biuret method [16].

Results

The time-course of the peroxidation process was studied in the light of formation of malondialdehyde. Figs. 1 and 2 show malondialdehyde production vs. concentration of Fe^{2+} and mitochondrial protein, respectively. The curves are characterized by initiation phases of varied length,

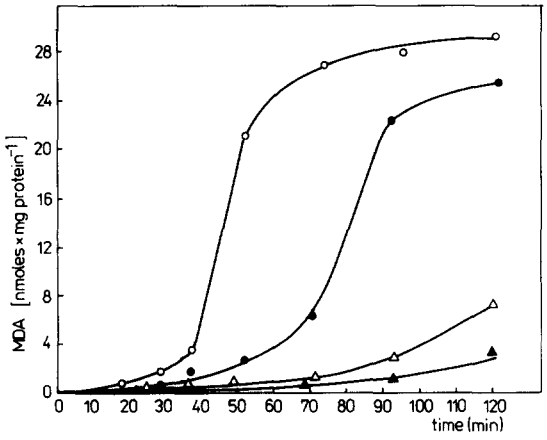

Fig. 1. Malondialdehyde (MDA) formation in rat liver mitochondria in dependence on various concentrations of Fe^{2+} for the initiation of lipid peroxidation 10 μM Fe^{2+} (▲———▲); 20 μM Fe^{2+} (△———△); 40 μM Fe^{2+} (●———●); 80 μM Fe^{2+} (○———○) in the presence of 6.6 mg mitochondrial protein/ml; other conditions as described in Materials and Methods.

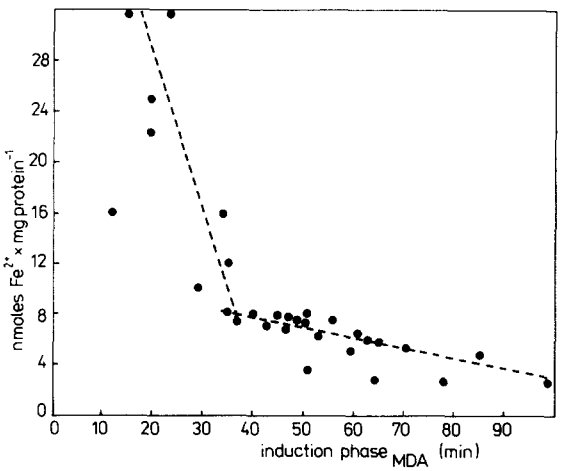

Fig. 3. Length of the initiation phase of malondialdehyde (MDA) formation vs. different Fe^{2+}/protein ratios. Induction $phase_{MDA}$ was obtained by the points of intersection of the two tangents (one each for the initial and the exponential phases of malondialdehyde formation). Data points represent results of 30 independent experiments of the type depicted in Figs. 1 and 2.

a rapid autocatalytic period, and different final levels of malondialdehyde. The time lag required until the onset of malondialdehyde formation was seen to highly depend on the Fe^{2+}/protein ratio in the incubation medium (Fig. 3). The critical Fe^{2+} concentration permitting a sufficient temporal resolution of the initiation phase of peroxidation was seen in the range from 4 to 8 nmol Fe^{2+} per mg mitochondrial protein. In this way, experiments may be suitably performed with different induction times of peroxidation so as to study early effects of oxidative stress on mitochondrial functions.

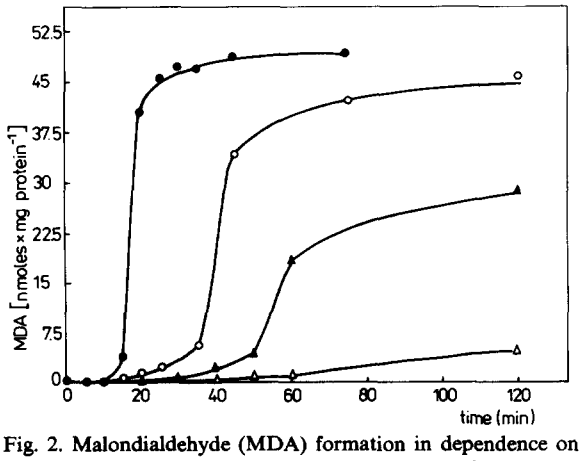

Fig. 2. Malondialdehyde (MDA) formation in dependence on the mitochondrial protein concentration: 1.25 mg/ml (●———●); 2.5 mg/ml (○———○); 5.0 mg/ml (▲———▲); 10.0 mg/ml (△———△); Fe^{2+} = 40 μM.

In many experiments, the non-sophisticated and sensitive thiobarbituric acid reaction was directly compared with the measurement of lipohydroperoxides the formation of which occurs somewhat earlier than that of malondialdehyde, but generally an identical time course was noted (not shown).

Fig. 4 depicts the effect of peroxidation induced by ferrous ions and ascorbate, on the active, controlled and FCCP-stimulated respiration. It is evident that, during the initiation phase of malondialdehyde formation, both the phosphorylating and the uncoupler-stimulated respiration were increasingly inhibited, whereas the resting state respiration remained almost unaffected. Hence, the diminishing respiratory control ratios resulted from a decrease in ADP-stimulated respiration rather than from an enhancement of controlled respiration. Furthermore, the increasing inhibition of the uncoupled respiration indicated that initial membrane alterations during peroxidation were not associated with an elevated proton leak. More likely, they were connected with an inhibition of the capacity of respiration. It was

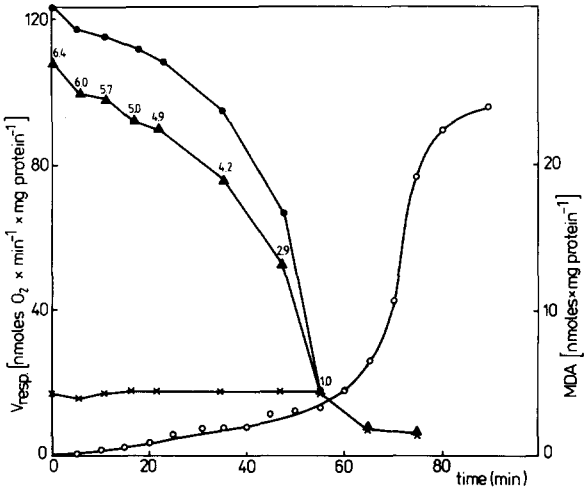

Fig. 4. Influence of peroxidation on the respiratory rate in functionally intact rat liver mitochondria; resting state respiration (× ——— ×), 10 mM succinate as substrate; active respiration (▲———▲), adjusted by 0.2 mM ADP, numbers represent respiratory control ratios; FCCP-stimulated respiration (0.1 μM FCCP) (●———●); malondialdehyde (MDA) formation (○ ——— ○). One representative experiment of ten, in the presence of 20 μM Fe^{2+} and 5.4 mg mitochondrial protein/ml.

established in sixteen independent experiments with both succinate and glutamate/malate that the length of the lag phases of malondialdehyde

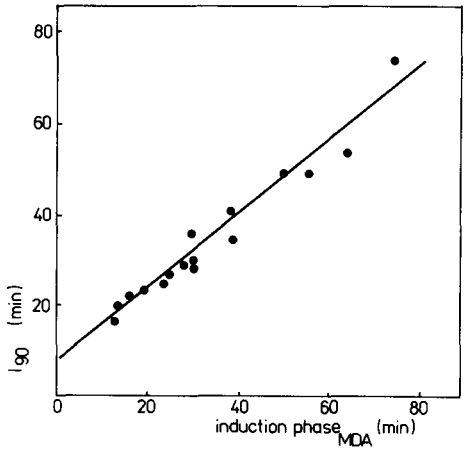

Fig. 5. Relationships between malondialdehyde (MDA) formation and inhibition of phosphorylating respiration. I_{90} denotes the time necessary to achieve 90% inhibition of active respiration. Experiments were performed according to Fig. 4 with succinate and glutamate plus malate as respiratory substrates. Ranges of respiratory rates were 85.5–106.0 and 63.4–72.5 nmol O_2 per min per mg protein for succinate and glutamate/malate, respectively. Induction $phase_{MDA}$ as in Fig. 3. Data points represent results of 16 different experiments.

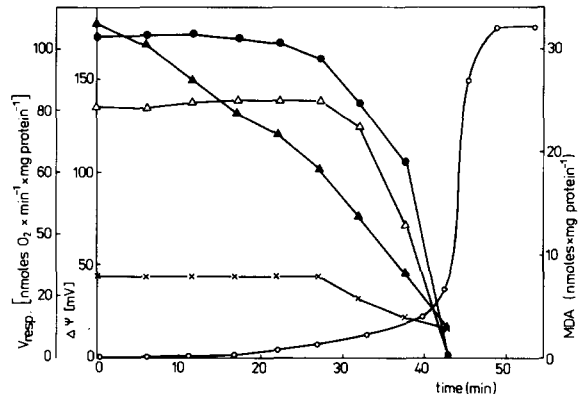

Fig. 6. Relations between respiratory rate, membrane potential and MDA formation during peroxidation; respiratory rate under phosphorylating conditions (▲———▲); respiratory rate under resting state conditions (× ——— ×); membrane potential under phosphorylating conditions (△———△); membrane potential under resting state conditions (●———●); malondialdehyde (MDA) formation (○ ——— ○); one typical experiment of five, with 33 μM Fe^{2+} and 5.6 mg mitochondrial protein/ml.

formation closely correlated ($r = 0.976$) with the time intervals needed to attain 90% inhibition of active respiration (Fig. 5). Thus, the phosphorylating respiration had been strongly inhibited even before considerable amounts of malondialdehyde formed.

TABLE I

SUMMARY OF RELATIONS BETWEEN ACTIVE RESPIRATION, MEMBRANE POTENTIAL, MALONDIALDEHYDE AND GLUTATHIONE CONTENT DURING Fe^{2+}/ASCORBATE-INDUCED PEROXIDATION IN RAT LIVER MITOCHONDRIA

The data represent arithmetic means ± S.D. of five experiments. Because of the differences in the time courses of the single incubations, the values of Δψ, malondialdehyde (MDA) and GSH were calculated on the basis of equal respiration rates. The Δψ values are related to phosphorylating conditions.

Respiration rate (nmol O_2 per min per mg protein)	Membrane potential (mV)	(nmol per mg protein)	
		MDA	GSH
90	137.8 ± 4.7	0	7.5 ± 0.9
85	139.4 ± 3.9	0.3 ± 0.2	7.1 ± 0.5
70	138.8 ± 5.4	0.8 ± 0.6	6.3 ± 0.7
50	133.4 ± 5.8	1.4 ± 0.9	5.2 ± 0.6
25	72.2 ± 6.1	2.5 ± 1.5	4.3 ± 0.6
20	42.0 ± 6.5	4.1 ± 2.6	3.3 ± 0.9
15	0	12.3 ± 4.5	1.9 ± 1.0

After initiation of peroxidation, respiratory rates and transmembrane potentials were measured simultaneously under both phosphorylating and resting state conditions (Fig. 6). The active respiration being continuously inhibited as shown above, the membrane potential was built up for a comparatively long period without undergoing substantial changes or with only minor decreases, and experiencing a fairly pronounced drop not until after the active respiration had been markedly inhibited. The interrelations of the parameters shown in Fig. 6 and Table 1 reveal that, in general, the onset of heavy malondialdehyde formation was preceded by diminishing respiration and membrane potential. These changes were also accompanied by a drop of mitochondrial GSH down to a level of about one-third of the normal content.

Discussion

It was the purpose of the present work to characterize early responses of mitochondrial functions during peroxidative attack on membranes. The degree of peroxidation was determined by measuring the accumulation of thiobarbituric acid-reactive substances, a convenient method which, while being easy to apply, is highly sensitive [13,17,18]. The pros and cons of malondialdehyde used as a marker for the extent of peroxidation reactions have been repeatedly discussed in the literature [17,19]. Even though malondialdehyde reflects breakdown products of peroxidized fatty acids, which are formed relatively late during the peroxidation process, other approaches to characterize lipid peroxidation did not provide suitable alternatives. The lipohydroperoxide assay or indirect measurements such as that of mitochondrial swelling closely correlated with malondialdehyde (not shown), but did not offer particular advantages.

As is clear from Figs. 1–3 the start of heavy malondialdehyde production is adjustable by selecting a suitable Fe^{2+}/protein ratio. Accurate adjustment, however, has not been possible to date, since the kinetics of malondialdehyde formation appears to be influenced by other factors not yet defined.

Under carefully controlled conditions it was unequivocally shown that at least the majority of the functional changes in isolated rat liver mitochondria occur prior to the onset of heavy lipid peroxidation as manifested by the start of exponential malondialdehyde formation. In other words, intensive lipid peroxidation can take place not until just after the breakdown of mitochondrial functions. In particular, this was demonstrated for the decrease in active respiration representing the functional parameter, which was most sensitively influenced in the time-course of Fe^{2+}/ascorbate-induced peroxidation. There seemed to be no striking influence exerted by the type of respiratory substrate, since both succinate as well as glutamate plus malate gave comparable results (data included in Fig. 5). Thus, the particular sensitivity of site I, but not site II, substrate oxidation to oxidative stress as suggested in Ref. 20 and attributed to the specific attack at the level of SH-groups (2-oxoglutarate and pyruvate dehydrogenases) was not confirmed in the present experiments. This may have been due to the different model systems employed (*t*-butyl hydroperoxide and Fe^{2+}/ascorbate, respectively).

In contrast to previous findings by Vladimirov and Cheremisina [2] reporting that uncoupling of oxidative phosphorylation and an increase in proton permeability occurred very early during Fe^{2+}/ascorbate-induced peroxidation when the respiratory chain was still unaltered [1], we found virtually no changes in the resting state respiration during the initiation phase of peroxidation. Probably, these controversial findings resulted from different experimental conditions; specifically, the concentrations of ferrous ions used in Ref. 2 were substantially higher and, hence, a temporal resolution of the initial events may not have been achieved. Judging from the present results, it is felt that the decrease in respiration be considered as the key event in the causal chain leading to the destruction of mitochondria. This suggestion is supported by the finding that, similarly, the respiratory rate measured in the presence of optimal uncoupler concentrations was inhibited with a kinetics that closely resembled that of phosphorylating respiration. Data pointing to primary impairment of mitochondrial respiratory activities have been reported recently for reperfused hypo-

thermic rat liver [21]. mitochondria from myocardium after reperfusion injury [22], and rat kidney mitochondria after ischaemia [23]. Whether in the experimental model used for the present investigations the decrease in active respiration was caused either directly by an attack at the level of the respiratory chain or indirectly by substrate access, is an open question which has to be elucidated in forthcoming studies.

The results obtained for the membrane potential in the course of peroxidation (Fig. 6, Table I) revealed that, regarding the sequence of events during peroxidative damage, no membrane alteration such as the occurrence of a proton leak or other ion leakage belonged to the primary phenomena. On the contrary, a normal-level membrane potential was built up in both the resting and the phosphorylating states at incubation times when respiratory rates already substantially decreased had been established. In the framework of the chemiosmotic coupling theory [24] it is not easy to understand that a nearly constant or only negligibly diminishing membrane potential can be sustained at phosphorylating respiratory rates which are reduced to about 50% of the initial values. Assuming that the decrease in respiration is compensated for by an impairment of membrane potential-utilizing processes, e.g., electrogenic transport steps, would seem to reasonably account for the fact that a high membrane potential is maintained in the early phase of peroxidation. Presumably, this balance is achieved by disconnection of potential consumers so as to keep the energy state of the membrane high enough to maintain membrane functions during peroxidizing conditions. Similar assumptions suggesting that, during anoxia of isolated hepatocytes, mechanisms may operate to minimize the collapse of the protonmotive force were made recently by Andersson et al. [25]. In the experimental model described in the present paper, the postulated mechanism(s) maintaining the membrane potential have to be classed into the group of defence systems against oxidative stress, whereas the final breakdown of membrane potential may be considered as the ultimate step before the onset of intensive lipid peroxidation. Rapid drops of mitochondrial membrane potential as observed during iron(III)-gluconate induced peroxidation by Masini et al. [5] may be attributed mainly to the high rate of peroxidative reactions due to the high concentration of iron ions. This would also account for the lack of a measurable initiation phase of malondialdehyde formation found in Ref. 5.

The membrane potential being maintained for a comparative long period under the present experimental conditions of iron/ascorbate-induced peroxidation may play an important role within the ensemble of protective mechanisms against mitochondrial injuries ensuing from oxidative stress. It is suggested from the present work that, being an important antioxidant system of mitochondria, GSH cannot be maintained under the conditions of oxidative stress induced by Fe^{2+}/ascorbate, and its decrease below a critical level is one of the prerequisites for the onset of intensive malondialdehyde formation.

Acknowledgements

The authors thank Prof. Dr. W. Kunz for helpful discussion. The skilful technical assistance of Mrs. K. Christoph is gratefully acknowledged.

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