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# LIPID PEROXIDATION IN EHRLICH ASCITES CELL MITOCHONDRIA IS NOT DETERMINED BY THE POLYUNSATURATED FATTY ACID CONTENT OF THE MEMBRANE+

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Lipid peroxidation intensity is compared in Ehrlich Ascites Cell and in liver mitochondria, prepared from tumour bearing mice. Malondialdehyde formation is negligible in intact ascites tumour mitochondria, but it is significantly increased in permeabilised mitochondria and in isolated mitochondrial membranes. We suggest that the resistance against oxidative stress is a consequence of efficient protective mechanisms operating in the intact tumour mitochondria and the low level of polyunsaturated fatty acids under these circumstances cannot be the rate limiting factor in lipid peroxidation. Succinate, an effective inhibitor of mitochondrial lipid peroxidation in liver, cannot determine malondialdehyde formation in ascites tumour mitochondria.


Lipid peroxidation seems to be associated with many physiological and pathological processes. A number of tumour cell lines have decreased susceptibility to oxidative stress in comparison to the corresponding normal tissues (1-3). A general hypothesis was put forward that increased rate of cell division is associated with a decreased rate of lipid peroxidation (4,5). Earlier it has been shown that in the mitochondria of Ehrlich ascites cells (EAC) ferrous ions (6) do not induce significant rate of malondialdehyde formation. The decreased rate of lipid peroxidation in tumour tissues has been explained by the decreased availability of polyunsaturated fatty acids (PUFA-s) in tumour membranes (5,6).

On the other hand, mitochondrial membranes isolated from normal cells contain high percentage of PUFA-s, therefore they are susceptible to peroxidative attack (7). Various defense systems have been described to

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maintain the structural and functional integrity of mitochondria under the circumstances of oxidative stress (8,9). In liver and heart mitochondria a succinate mediated free radical scavenger system has been described (10-12). This mechanism might play a role under pathological circumstances as well (13).

In the present study some characteristics of EAC and liver mitochondrial lipid peroxidation are compared to establish the factor(s) responsible for the low rate of malondialdehyde formation in ascites mitochondria. The importance of succinate-mediated lipid peroxidation inhibition in the tumour mitochondria is also investigated.

## MATERIALS AND METHODS

$CDF_1$ male mice were used throughout the experiments. Liver and ascites mitochondria were prepared as described (14,15). Mitochondrial inner membrane fraction was prepared from mitoplasts obtained by the digitonin method (16), mitoplasts were disintegrated with an MSE 100W sonic oscillator at 7μm amplitudes . The inner membrane fraction was sedimented at 105,000xg in a Beckman L2-65 ultracentrifuge for 60 min, washed twice and resuspended in 0.15M KCl and 20mM TRIS-HCl buffer, pH 7.2. Lipid peroxidation protocols: ADP/Fe/NADPH - 2mM ADP, 20μM $Fe_2(SO_4)_3$, 0.3mM NADPH. ADP/Fe/ascorbate - 2mM ADP, 20μM $Fe_2(SO_4)_3$ , 0.2mM ascorbate. Cumene hydroperoxide - 0.5mM cumene hydroperoxide. Incubation was performed in a 0.15M KCl, 20mM TRIS-Hcl buffer, at 37°C, pH 7.2. The rate of lipid peroxidation was estimated by measuring the formation of malondialdehyde (17). Succinate oxidase activity was measured by following the oxygen uptake with a Clarke type oxygen electrode (18). Proteins were determined by the modified biuret method (19).

## RESULTS

The lipid peroxidative capacity of EAC mitochondria was measured and found that significantly less malondialdehyde was produced by the tumour cell mitochondria than by the liver mitochondria of the host animals. As it is shown (Fig. 1.), this effect was independent on the nature of peroxidative stimuli. ADP/Fe/NADPH, ADP/Fe/ascorbate or cumene hydroperoxide elicited similar response.

The malondialdehyde formation of mitochondria is shown in Table I after they were made permeable with various agents and treatments . It can be seen that the disruption of mitochondria did not have significant influence on the lipid peroxidative capacity of liver mitochondria (Table I/A). On the other hand the increase of permeability of EAC mitochondria led to enhanced lipid peroxidation (Table I/B). It is known that non-ionic detergents may contain oxidizing impurities (20,21). In order to eliminate this possible artifact Lubrol WX and Triton X-100 were purified as described in (21). Addition of detergents to mitochondria without further peroxidative stimulus did not increase malondialdehyde formation (data not shown).

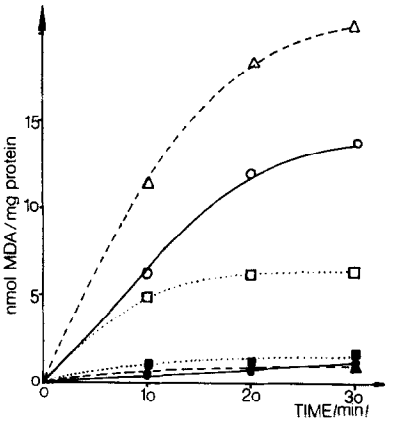


Fig.1.
LIPID PEROXIDATION IN LIVER AND ASCITES CELL MITOCHONDRIA INDUCED BY DIFFERENT MECHANISMS.
Malondialdehyde (MDA) formation in liver mitochondria induced by (○—○) ADP/Fe/NADPH; (△—△) ADP/Fe/ascorbate; (□··□) cumene hydroperoxide. MDA formation in Ehrlich ascites cell mitochondria induced by (●—●) ADP/Fe/NADPH; (▲—▲) ADP/Fe/ascorbate; (■··■) cumene hydroperoxide. Incubation was performed as described in Materials and Methods, using 2mg/ml mitochondrial protein. Results are expressed as mean of at least four experiments.

Table I. THE EFFECT OF MEMBRANE PERMEABILISATION ON ADP/Fe/NADPH DEPENDENT LIPID PEROXIDATION OF MITOCHONDRIA[a]

| | Time of incubation | | | |
|---|---|---|---|---|
| | 0 min | 10 min | 20 min | 30 min |
| | (nmol malondialdehyde/mg protein) | | | |
| A) LIVER MITOCHONDRIA | | | | |
| Intact mitochondria | nd. | 6.50 | 11.42 | 13.66 |
| Freeze-thawing 1X | nd. | 7.39 | 12.99 | 14.56 |
| Freeze-thawing 5X | nd. | 7.39 | 12.54 | 14.56 |
| Sonication[b] | 0.128 | 9.30 | 13.22 | 15.46 |
| B) ASCITES MITOCHONDRIA | | | | |
| Intact mitochondria | nd. | 0.16 | 0.34 | 0.78 |
| Freeze-thawing 1X | nd. | 0.22 | 0.45 | 1.12 |
| Freeze-thawing 5X | nd. | 0.27 | 0.78 | 1.64 |
| Sonication[b] | nd. | 0.45 | 1.34 | 1.90 |
| Triton X-100 0.1%[c] | nd. | 0.29 | 0.45 | 1.79 |

[a] Mitochondria, 2mg/ml protein, were incubated with 2.0mM ADP, 20μM $Fe_2(SO_4)_3$ and 0.3mM NADPH at 37°C.
[b] Mitochondria, 10 mg/ml protein, were sonicated for 20 sec x 3 by an MSE 100W sonic oscillator, 7μm amplitudes, at 0°C, then were diluted to 2mg/ml protein concentration.
[c] Detergent was added to the mitochondrial suspension (2mg/ml protein) at 0°C, 10 minutes before lipid peroxidation was started by adding ADP/Fe/NADPH.

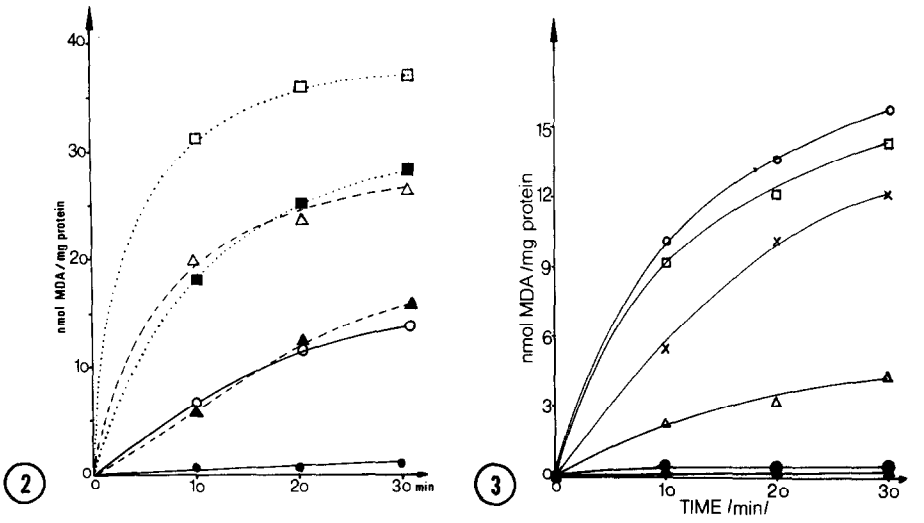


Fig.2.
LIPID PEROXIDATION OF MITOCHONDRIAL INNER MEMBRANES.
Malondialdehyde (MDA) formation was induced: in the inner membrane (1mg/ml protein) of liver mitochondria by (△-△) ADP/Fe/NADPH; (□···□) ADP/Fe/ascorbate, in the inner membrane of Ehrlich ascites cell (EAC) mitochondria by (▲-▲) ADP/Fe/NADPH; (■···■) ADP/Fe/ascorbate.
ADP/Fe/NADPH induced lipid peroxidation of intact liver (○—○) and EAC mitochondria (●—●) are shown for reference (2mg/ml protein). Results are expressed as mean of at least four experiments.

Fig.3.
THE EFFECT OF SUCCINATE AND THEONYLTRIFLUOROACETONE (TTFA) ON ADP/Fe/NADPH INDUCED LIPID PEROXIDATION OF LIVER AND EAC MITOCHONDRIA.
Malondialdehyde (MDA) formation was induced: in liver mitochondria (×—×); liver mitochondria plus 5mM succinate (△—△); liver mitochondria plus 10uM TTFA (○—○); liver mitochondria plus 5mM succinate plus 10uM TTFA (□—□). MDA formation in EAC mitochondria (●—●); EAC mitochondria plus 10$\mu$M TTFA (▲—▲). 2mg/ml protein concentration was used. Results are expressed as mean of at least four experiments.

In Fig. 2 it is shown that mitochondrial membranes are very sensitive to lipid peroxidation. The rate of lipid peroxidation in membranes isolated from ascites mitochondria is ten to twenty times higher than in intact mitochondria. In case of liver mitochondria this effect is less pronounced. Malondialdehyde formation in membranes was not further elevated by addition of Triton X-100 or Lubrol WX (not shown). From these experiments it is concluded that during disruption of mitochondria some parts of the protective mechanisms can be lost.

To investigate the role of substrate oxidation in the protection of mitochondria against lipid peroxidation, the succinate mediated lipid peroxidation inhibition was also studied. Succinate inhibits lipid peroxidation both in intact mitochondria and in mitochondrial inner membranes (14). In order to inhibit lipid peroxidation, succinate must be oxidized in the respiratory chain. Using theonyltrifluoroacetone (TTFA), the specific inhibitor of succinate dehydrogenase (E.C. 1.3.99.1), increase of MDA

formation was detected in liver mitochondria, but lipid peroxidation was not induced in ascites mitochondria (Fig.3.).It is also shown, that TTFA was able to suspend succinate-mediated lipid peroxidation inhibition in liver mitochondria. TTFA inhibition of succinate oxidase activity was higher than 92 per cent at $10^{-5}$M concentration (data not shown). In isolated inner membranes of mouse liver mitochondria the succinate inhibition of lipid peroxidation was about 50 per cent, but in ascites mitochondrial membranes the inhibition was insignificant (data not shown).

## DISCUSSION

In general, mitochondrial or microsomal suspensions prepared from cancer cells peroxidise slowly as compared to normal cells (4,5). There are a number of possible reasons for such a decreased rate of lipid peroxidation. Firstly, the enzyme system, taking part in the generation of free radicals might have decreased activity in tumour cells. Secondly, the content of PUFA-s in the tumour membrane phospholipids may be reduced. Thirdly, the protective systems against free radical damage may have increased activity.

Three types of lipid peroxidation were investigated in EAC mitochondria (Fig.1). Induction of lipid peroxidation by ADP/Fe/ascorbate and cumene hydroperoxide does not need enzymic components (22), and they were not able to induce lipid peroxidation, indicating that the lack of lipid peroxidation can not be explained by the decreased activity of free radical generating enzyme(s) in EAC mitochondria.

In earlier reports, the lack of malondialdehyde formation in ascites tumour mitochondria was attributed only to the decreased availability of PUFA-s for lipid peroxidation and malondialdehyde formation (6,5). This view was supported by experiments on radiation-induced lipid peroxidation in liposomes (23). The incorporation of non-peroxidizable lipids into liposomes reduced the rate of malondialdehyde formation. However, we have now shown that the increase of permeability of ascites mitochondrial membranes raised MDA formation from two to four times (Table I.). The lipid peroxidation of liver mitochondria did not change considerably. It has been described (24), that during the course of lipid peroxidation liver mitochondria became permeable to large molecules, even for proteins.

This implies, that important parts of the antioxidant system could be released from liver mitochondria even after ten minutes incubation. It is not surprising then, that in liver mitochondria the artificial disruption of membrane integrity does not elevate considerably the intensity of lipid peroxidation, because components taking part in the free radical scavenging system could be released from the mitochondria without sonication or detergent treatment as well.

Using isolated mitochondrial inner membranes the malondialdehyde formation was further elevated especially in EAC membranes. As, it is unlikely that the amount of highly unsaturated fatty acids would be increased during the membrane isolation process, the insensitivity of intact ascites mitochondria can not be explained solely by the low PUFA concentration in the membrane. Therefore, the most likely explanation for the low rate of lipid peroxidation is the existence of effective scavenger system(s) in the ascites mitochondria. It is supposed, that due to membrane permeabilisation or membrane preparation important components of the free radical scavenger system were lost, or disconnected. Under these circumstances (in isolated membranes) probably the PUFA content is the rate limiting factor in lipid peroxidation.

In our earlier reports (12,14,24) the role of succinate in preventing mitochondrial lipid peroxidation was investigated. Therefore, we studied the possible involvement of succinate in the inhibition of lipid peroxidation of EAC mitochondria. Fig.3 shows, that using theonyltrifluoroacetone (TTFA), a specific inhibitor of succinate dehydrogenase, lipid peroxidation was increased in liver mitochondria, indicating that the oxidation of endogenous substrates might protect mitochondria to certain extent against oxidative stress. It is also shown, that TTFA was able to suspend the inhibition of succinate in liver mitochondria, but it was not able to increase malondialdehyde formation in ascites mitochondria. In the inner membranes of ascites mitochondria, the succinate mediated inhibition was negligible, but about 50 per cent inhibition was found in liver membranes (data not shown). These data indicate that succinate mediated lipid peroxidation inhibition probably could not play a determining role in the modulation of lipid peroxidation in EAC mitochondria. The role of other soluble and membrane bound free radical scavenging systems in EAC mitochondria remains to be investigated.

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