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THE INVOLVEMENT OF SEMIDEHYDROASCORBATE REDUCTASE IN THE OXIDATION OF NADH BY LIPID PEROXIDE IN MITOCHONDRIA AND MICROSOMES

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SUMMARY

1. A mechanism for the oxidation of NADH by a lipid peroxide is described.
2. The reaction, which is dependent on the presence of ascorbate, occurs in both the mitochondrial and microsomal fractions.
3. Due to several observed similarities to the characteristics of semidehydroascorbate reductase (reduced-NAD(P):oxidized-ascorbate oxidoreductase, EC 1.6.5.4) the involvement of this enzyme in the reaction pathway is proposed.
4. It is proposed that a reaction between peroxide and ascorbate produces the free-radical intermediate semidehydroascorbate. This intermediate is then reduced by NADH to ascorbate in a reaction catalyzed by semidehydroascorbate reductase.
5. The importance of this pathway for the removal of potentially harmful peroxides is discussed.
6. Mitochondria can also catalyze the oxidation of NADH by lipid peroxide by a different mechanism which does not involve ascorbate.

INTRODUCTION

Lipid peroxides can be produced intracellularly under a variety of conditions[1–3]. Accumulated lipid peroxides, which cannot be decomposed by catalase[4], lead to a number of changes harmful to the cell. It is therefore feasible to assume that mechanisms exist for the detoxification of lipid peroxide. Peroxides can be decomposed in the presence of metal ions or heme compounds[5], but this mechanism probably involves production of more harmful free-radicals[6]. Lipid peroxide can be reduced by NADPH

Abbreviation: LAHPO, linoleic acid hydroperoxide.

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in reactions involving the glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) and glutathione reductase (reduced-NAD(P):oxidized-glutathione oxidoreductase, EC 1.6.4.2) of the cytosol. However, the accumulation of lipid peroxide in particulate fractions occurs more readily due to the presence of unsaturated fatty acids within the organelle membranes. Also in the microsomal fraction there exists an NADPH-dependent enzyme system which catalyzes the peroxidation of microsomal lipids[8].

In the following a reaction sequence is described whereby lipid peroxide can be reduced by NADH in the presence of ascorbate. The enzyme semidehydroascorbate reductase (reduced-NAD(P):oxidized-ascorbate oxidoreductase, EC 1.6.5.4) appears to be involved. This system is operative in both the mitochondrial and microsomal fractions from liver. A preliminary report of this work has been presented[9].

MATERIALS AND METHODS

An ethanolic solution of linoleic acid hydroperoxide (LAHPO) was prepared as described by O'Brien[5] and stored at −20 °C. Immediately prior to use, the ethanol was removed by a stream of N_2, the LAHPO was neutralized with one equivalent of NaOH and made up to volume with water containing 10 μM EDTA. Solutions of ascorbic acid (Nutritional Biochemicals) were kept at 0 °C and neutralized just before use. Dehydroascorbate was supplied by Schwarz/Mann; all other reagents were of analytical grade.

Subcellular fractions

A 10% (w/v) homogenate of rat liver in 0.3 M sucrose containing 2 mM EDTA was prepared in a Teflon-glass homogenizer. Nuclei and debris were removed at 600 × *g* for 10 min. The mitochondria were sedimented at 7500 × *g* for 10 min, washed twice in sucrose–EDTA, and then resuspended in the same medium. The 7500 × *g* supernatant was centrifuged at 20 000 × *g* for 20 min to remove the heavy mitochondria and lysosomes which were discarded.

Finally, the microsomal pellet was sedimented at 105 000 × *g* for 1 h and resuspended in sucrose–EDTA.

Just prior to use the stock suspension of mitochondria (10 mg protein/ml) was titrated at 0 °C with a 5% (v/v) solution of Triton X-100 in order to bring about membrane disruption. As the critical concentration of Triton was reached, the appearance of the suspension rapidly changed from totally opaque to quite clear. The final concentration of Triton required was about 0.05% (v/v). It was not possible to use untreated or even sonicated mitochondria in experiments involving LAHPO since a steady decrease in absorbance caused by the detergent effect of the fatty acid peroxide on the membranes made it impossible to use spectrophotometric methods. The microsomal fraction was similarly treated prior to use but the end-point was not as clear.

Semidehydroascorbate reductase

The activity was measured at 25 °C by following the oxidation of NADH in a system containing 70 mM Tris–HCl, pH 7.5; 1.0 mM EDTA; 0.25 mM KCN; 10.0

mM ascorbate; 10.0 mM dehydroascorbate and 100 μM NADH. Other analytical methods are described in the legends to figures and tables.

RESULTS

Cyanide-sensitive system

Before discussing the role of ascorbate in the oxidation of NADH by peroxide it is necessary to describe the reactions which occur in the absence of ascorbate.

TABLE I

THE OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES BY LAHPO, CATALYZED BY MITOCHONDRIA

The reaction mixture contained, in a final volume of 3.0 ml, 70 mM Tris–HCl (pH 7.5); disrupted mitochondria, 2 mg protein/ml; EDTA 1 mM; LAHPO, 0.35 mM; NADH or NADPH, 0.1 mM. The decrease in $A_{340\ nm}$ due to oxidation of the reduced nucleotide was followed with a recording spectrophotometer.

Treatment	*Rate of oxidation of NADPH (nmoles/min)*	*Rate of oxidation of NADH (nmoles/min)*
None*	12	91
None	14	149
Boiling	—	0
KCN, 50 μM	—	20

* No LAHPO present.

Triton-disrupted mitochondria are capable of catalyzing the oxidation of reduced pyridine nucleotides (Table I). This preparation oxidized NADH about eight times faster than NADPH. The addition of LAHPO caused approximately a 65% increase in the rate of NADH oxidation but only a slight increase in NADPH oxidation. Boiled mitochondria did not catalyze the oxidation of NADH by LAHPO, and it therefore appears that an enzyme or heat-sensitive system is involved. The oxidation of NADH, both in the absence and presence of LAHPO, was strongly inhibited by 50 μM KCN. In the absence of mitochondria, no oxidation of NADH by LAHPO was observed.

It is known that the oxidizing capabilities of peroxides can be stimulated by a number of heme-containing compounds[10]. It is unlikely that such a non-specific mechanism can account for the phenomenon observed with mitochondria, however, since the hemoprotein-catalyzed reaction is not sensitive to boiling. Furthermore, although hemoglobin by itself catalyzed the oxidation of NADH by LAHPO, the addition of hemoglobin to the mitochondrial system decreased the rate of oxidation of NADH by LAHPO.

In the microsomal fraction, the addition of LAHPO produced only a slight increase in the rate of NADH oxidation. Furthermore, NADH oxidation in this subcellular fraction was only slightly inhibited by cyanide.

The ascorbate-catalyzed oxidation of NADH by LAHPO

It was demonstrated (Fig. 1) that, in the absence of any subcellular fraction, ascorbate protected NADH from the hemoprotein-catalyzed oxidation by LAHPO.

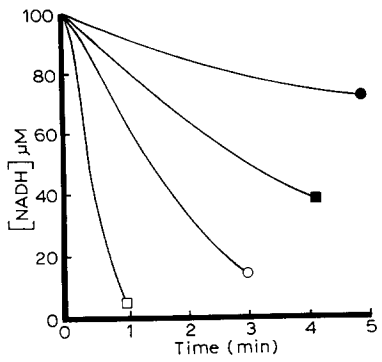


Fig. 1. Effect of ascorbate on the hemoglobin-catalyzed oxidation of NADH by LAHPO. The reaction mixture contained Tris–HCl, 70 mM (pH 8.5); methemoglobin, 50 μg/ml; LAHPO, 0.6 mM; NADH, 0.1 mM. The oxidation of the NADH was followed on a recording spectrophotometer at 340 nm in the presence of different concentrations of ascorbate. □, no ascorbate; ○, 20 μM ascorbate; ■, 100 μM ascorbate; ●, 200 μM ascorbate.

TABLE II

EFFECT OF ASCORBATE ON THE OXIDATION OF NADH BY LAHPO IN SUBCELLULAR FRACTIONS

The assay medium consisted of Tris–HCl, 70 mM (pH 7.5); EDTA, 1 mM; subcellular fraction, 0.3–1.0 mg protein/ml; NADH, 0.1 mM.

Addition	*Rate of NADH oxidation (nmoles/min per mg protein)*		
	Mitochondrial	*Microsomal*	*Supernatant*
None	51	26	0
Ascorbate, 2 mM	51	26	0
LAHPO, 0.33 mM	85	29	14
LAHPO, 0.33 mM + ascorbate, 2 mM	162	78	14

The possibility that in subcellular fractions the oxidation of NADH by LAHPO would be inhibited by ascorbate was therefore investigated. However, as seen in Table II, ascorbate produced a marked increase in the rate of NADH oxidation by LAHPO in the mitochondrial and microsomal fractions and was without effect in the supernatant fraction. No ascorbate-induced stimulation was observed for the oxidation of NADPH by LAHPO in either the mitochondrial or microsomal fractions. In the supernatant fraction, ascorbate produced an inhibition of the oxidation of NADPH by LAHPO.

In the absence of peroxide, ascorbate had no stimulatory effect on the oxidation of NADH in either particulate fraction nor did the interaction between LAHPO and ascorbate produce any change in the absorbance at 340 nm in the absence of NADH.

The increase in the rate of NADH oxidation by LAHPO upon addition of ascorbate was not affected by the presence of cyanide in either the mitochondrial or microsomal fractions. This enabled the ascorbate-dependent oxidation to be studied more easily since cyanide could be used to inhibit the background oxidation of NADH in mitochondria. Measurements were more difficult to make accurately using

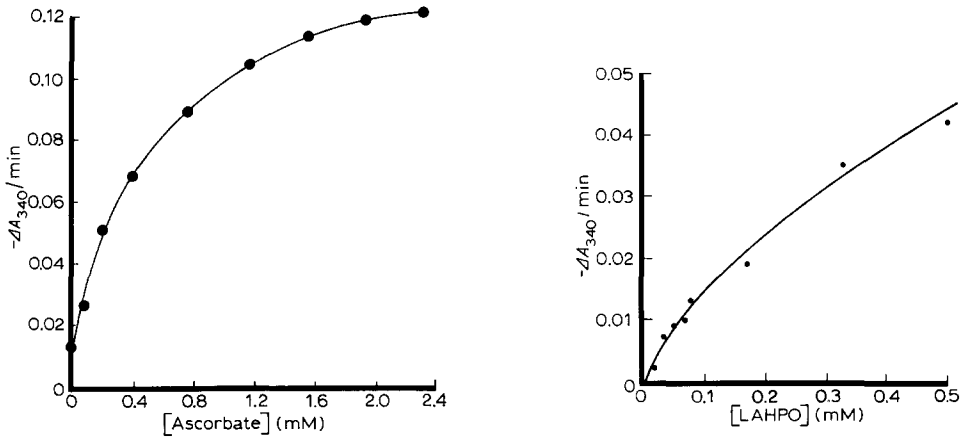


Fig. 2. Effect of ascorbate on the mitochondria-catalyzed oxidation of NADH by LAHPO The reaction mixture contained Tris–HCl, 70 mM (pH 7.5); EDTA, 1 mM; KCN, 0.25 mM; LAHPO, 0.66 mM; NADH, 0.1 mM; Triton-treated mitochondria, 0.33 mg protein/ml. Ascorbate was added last and the oxidation of NADH followed at 340 nm.

Fig. 3. Ascorbate-catalyzed oxidation of NADH by LAHPO. The reaction mixture contained Tris–HCl, 70 mM (pH 7.5); EDTA, 1.0 mM; KCN, 0.25 mM; NADH, 0.1 mM; Triton-treated mitochondria, 0.33 mg protein/ml. The rate of NADH oxidation after addition of ascorbate 1.9 mM, to the cuvette was measured. The slight rate of oxidation in the absence of ascorbate was subtracted.

the microsomal fraction since even in the presence of cyanide, the background oxidation of NADH was relatively fast.

The difference in sensitivity to cyanide demonstrates that, in mitochondria the ascorbate-dependent oxidation of NADH by LAHPO proceeds by a mechanism quite different from that which is responsible for the oxidation which occurs in the absence of ascorbate.

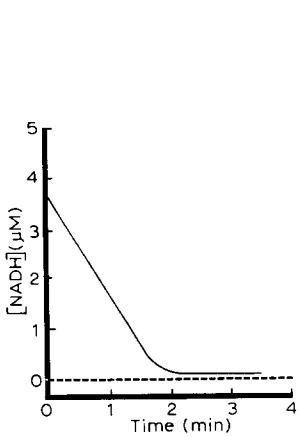
Kinetics of the ascorbate-dependent reaction

Fig. 2 shows the effect of increasing ascorbate concentration on the oxidation of NADH by LAHPO. Cyanide was included in the reaction mixture in order to inhibit the ascorbate-independent oxidation. The reaction shows saturation-type kinetics with respect to ascorbate. It was observed that the time curves were linear even at the lowest ascorbate concentration, 0.08 mM.

Fig. 3 shows the effect of increasing the concentration of LAHPO. In the absence of ascorbate there was little effect due to the presence of cyanide, but with ascorbate present the rate of oxidation increased as the LAHPO concentration was raised. The reaction appears essentially first order with respect to LAHPO. The deviation observed at LAHPO concentrations of 0.5 mM and higher is probably due to inactivation of the catalysts by the peroxide. At low LAHPO concentrations the time curves were non-linear. The rate of oxidation of NADH decreased as the LAHPO was consumed. This is consistent with the role of LAHPO as a substrate in the reaction. However, since it was observed that when using a high concentration of LAHPO (0.66 mM) the time curves were linear even at the lowest ascorbate concentration (where the concentration of ascorbate was lower than that of the NADH)

this indicates that there was no net decrease in the level of ascorbate during the course of the reaction. The ascorbate appears, therefore, to be involved in a catalytic role as a cofactor or to be involved in a reaction sequence whereby it is continually recycled.

An estimate of the K_m for NADH for its ascorbate-dependent oxidation by LAHPO was made by following the course of oxidation of NADH on a recording spectrophotometer with a full-scale deflection of 0.1 absorbance units. An extinction coefficient of $E_{340}^{mM} = 6.3$ was used. It was observed that the time curve (Fig. 4) was linear until the concentration of NADH had fallen below 5 μM, at which point the concentration of NADH became the rate-limiting factor. The rate of NADH oxidation decreased to half its original (maximal) value only below an NADH concentration of 1.0 μM. The K_m for NADH was thus less than 1.0 μM and was estimated to be about 0.3 μM. A more precise measurement of the K_m using this technique is difficult to obtain since an NADH concentration of 1.0 μM corresponds to an absorbance of only $6 \cdot 10^{-3}$ units.



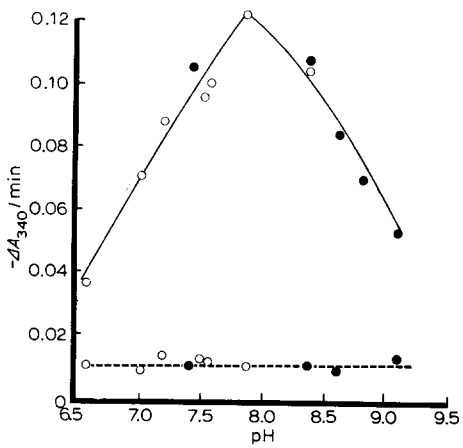


Fig. 4. Time curve for the ascorbate-catalyzed oxidation of NADH by LAHPO. The reaction mixture contained Triton-treated mitochondria, 0.05 mg protein/ml; Tris–HCl, 70 mM (pH 7.5); EDTA, 1 mM; KCN, 0.25 mM; LAHPO, 0.66 mM; ascorbate, 2.0 mM; and NADH, 4 μM. The decrease in absorbance at 340 nm was measured in a Unicam SP800 spectrophotometer, with a 0–0.1 A scale expansion accessory and external chart recorder.

Fig. 5. Effect of pH on the ascorbate-dependent oxidation of NADH by LAHPO. The assay medium contained Tris–HCl, 70 mM, or potassium phosphate buffer, 70 mM; EDTA, 1 mM; KCN, 0.25 mM; NADH, 0.1 mM; LAHPO, 0.66 mM. The decrease in absorbance at 340 nm was measured before and after the addition of 1.52 mM ascorbate. The pH was measured at the end of the reaction. ○, phosphate buffer; ●, Tris–HCl buffer. — — —, no ascorbate; ———, after addition of ascorbate.

The ascorbate-dependent oxidation of NADH by LAHPO was approximately first order with respect to protein concentration. Some deviation at high protein concentration (above 0.4 mg/ml) was observed when substrate concentrations became rate limiting.

As can be seen from Fig. 5, the ascorbate-dependent reaction exhibits a maximal rate at a pH of about 7.8.

Comparison with semidehydroascorbate reductase

The possible involvement in the reaction of the enzyme semidehydroascorbate reductase was next investigated. Using the assay system described in the Materials and Methods section, the time curve of the reductase reaction was followed and the K_m for NADH was estimated at 0.3 μM, the same value obtained for the ascorbate-dependent oxidation of NADH by LAHPO. It was found that both the reductase activity and the ascorbate-dependent oxidation of NADH by LAHPO were totally inhibited by 30-s treatment of the mitochondrial preparation with 0.1 mM *p*-chloromercuribenzoate. Addition of the mercurial to the assay system without preincubation had little effect on the oxidation, indicating that substrates might protect the enzyme. It was then found that the presence of NADH or NAD^+ during the pre-incubation with the inhibitor gave complete protection.

Another similarity between the two systems was the equal effectiveness of both α-NADH and β-NADH in both cases.

TABLE III

COMPARISON OF THE RESPONSE OF SEMIDEHYDROASCORBATE REDUCTASE AND THE ASCORBATE-DEPENDENT OXIDATION OF NADH BY LAHPO TO METAL-BINDING AGENTS

Semidehydroascorbate reductase was assayed as described in the Materials and Methods section. The ascorbate-dependent oxidation of NADH by LAHPO was assayed by measuring the increase in the rate of NADH oxidation that occurred at 25 °C upon addition of ascorbate, 5.0 mM, to a mixture containing potassium phosphate, 0.1 M (pH 7.5); LAHPO, 0.5 mM; NADH, 0.1 mM, and Triton-treated mitochondria, 0.3 mg protein/ml. Inhibitors were preincubated with the buffered mitochondria for 30 s at 25 °C before other reagents were added.

Addition	*Semidehydroascorbate reductase (%)*	*Ascorbate-dependent oxidation of NADH by LAHPO (%)*
None	100	100
o-Phenanthroline, 0.1 mM	100	10
8-hydroxyquinoline, 0.1 mM	100	80
Neocuproine, 0.1 mM	100	70
EDTA, 1 mM	90	150

Treatment of the mitochondrial preparation with metal-binding agents produced different results on the two activities (Table III). Little effect on the reductase activity was observed but the ascorbate-dependent oxidation of NADH by LAHPO was inhibited by *o*-phenanthroline, 8-hydroxyquinoline and neocuproine which are compounds capable of interacting with metal-containing proteins[11]. Since EDTA did not inhibit the reaction, it is unlikely that free metal ions are responsible. Also the addition of 0.1 mM of either $CuSO_4$ or $FeCl_2$ had no stimulating effect on the ascorbate-dependent oxidation of NADH by LAHPO. Possibly therefore a metalloprotein is involved.

DISCUSSION

Staudinger and co-workers[12–15] have described an enzyme system, occurring in the mitochondrial and microsomal fractions from liver and kidney, which can catalyze the oxidation of NADH in the presence of ascorbate and O_2. The species

directly responsible for this oxidation appears to be the intermediate in the oxidation of ascorbate to dehydroascorbate, namely, semidehydroascorbate. This free-radical species is also produced during the reaction between ascorbate and electron acceptors other than O_2, such as ferricytochrome, *c*, hexacyanoferrate (III) and 2,6-dichlorophenol indophenol[16]. Hence it is quite possible that the reaction between lipid peroxide and ascorbate could give rise to the semidehydroascorbate radical. Subsequent oxidation of the NADH is then accomplished by semidehydroascorbate reductase, with the concomitant regeneration of ascorbate.

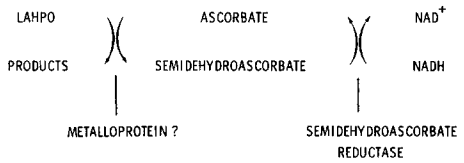


Fig. 6. Mechanism of the ascorbate-dependent oxidation of NADH by LAHPO.

The involvement of semidehydroascorbate reductase in the ascorbate-dependent oxidation of NADH by LAHPO, summarized in Fig. 6, is supported by the observations reported. The net level of ascorbate does not appear to change during the course of the reaction. The subcellular distribution of the two systems is the same. The observed pH optimum of 7.8 compares with the figure of 7.4 quoted by Lumper *et al.*[12] for the pH optimum of the mitochondrial semidehydroascorbate reductase. Both activities are sensitive to the mercurial *p*-chloromercuribenzoate. The K_m of NADH for both reactions is approximately 0.3 μM, and both reactions can also utilize α-NADH. The ability of an enzyme to accept both β- and α-NADH is unusual.

The exact nature of any catalyst involved in the first step of the reaction, that between LAHPO and ascorbate, cannot be ascertained from these experiments. However the inhibitory effect of the metal-binding reagents, which were without effect on semidehydroascorbate reductase, indicates that a metalloprotein may be involved in this first step. It is possible that a non-specific catalyst could be involved, namely one or more of the metal-containing proteins present in the mitochondrial and microsomal electron transport systems. The kinetics of such a mechanism would be first order with respect to peroxide[17]. Alternatively the data in Fig. 3 could be interpreted as being due to the involvement of a specific enzyme catalyst with a high K_m for LAHPO.

The overall reaction characteristics appear to be very similar in both the mitochondrial and microsomal fractions. It now appears that the semidehydroascorbate reductase activity involves one or more components of the NADH-linked microsomal electron transport system[18]. A similar electron transport system is located in the mitochondrial outer membrane[19]. Semidehydroascorbate reductase activity is also localized in the outer membrane[18] and it is therefore likely that the ascorbate-dependent oxidation of NADH by LAHPO would also occur in the outer membrane of the mitochondrion.

This pathway could provide an effective mechanism for the removal of potentially harmful lipid peroxides from the cell by utilizing the reductive capability of NADH. Its effectiveness would depend on the levels of ascorbate available within

the cell. No other complete mechanism for the destruction of lipid peroxide has been found in mitochondria, since the glutathione peroxidase pathway would be dependent on extra-mitochondrial GSH and GSSG reductase.

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