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NADPH-DEPENDENT INHIBITION OF LIPID PEROXIDATION IN RAT LIVER MICROSOMES

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Microsomal NADPH-driven electron transport is known to initiate lipid peroxidation by activating oxygen in the presence of iron. This pro-oxidant effect can mask an antioxidant function of NADPH-driven electron transport in microsomes via vitamin E recycling from its phenoxyl radicals formed in the course of peroxidation. To test this hypothesis we studied the effects of NADPH on the endogenous vitamin E content and lipid peroxidation induced in liver microsomes by an oxidation system independent of iron: an azo-initiator of peroxyl radicals, 2,2'-azobis (2,4-dimethylvaleronitrile), (AMVN), in the presence of an iron chelator deferoxamine. We found that under conditions NADPH: (i) inhibited lipid peroxidation; (ii) this inhibitory effect was less pronounced in microsomes from vitamin E-deficient rats than in microsomes from normal rats; (iii) protected vitamin E from oxidative destruction; (iv) reduced chromanoxyl radicals of vitamin E homologue with a 6-carbon side-chain, chromanol-α-C-6. Thus NADPH-driven electron transport may function both to initiate and/or inhibit lipid peroxidation in microsomes depending on the availability of transition metal catalysts.

"Enzymic" lipid peroxidation activated by NADPH and ADP-Fe^{3+} in rat liver microsomes was first described by Hochstein and Ernster more than 25 years ago [1]. Since then initiation of lipid peroxidation by NADPH and iron-chelates was discovered in microsomal and mitochondrial fractions from different normal tissues (liver, brain kidney, testis, placenta, lymphocytes, etc.), as well as in tumor cells [2-4]. The role of NADPH-cytochrome P-450 reductase and cytochrome P-450 in initiating lipid peroxidation by oxygen activation has been extensively studied during the past decade [5,6]. It was generally believed that NADPH cytochrome P-450 reductase was mainly involved in the process. However, recent reports indicated that both enzymes participate in the microsomal lipid peroxidation by providing two electrons for O_2 reduction [7].

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The NADPH-induced accumulation of lipid peroxidation products and oxidative loss of polyunsaturated phospholipids in membranes has been shown to be preceded by the consumption of endogenous antioxidants, in particular of vitamin E [8-10]. Recently the enzymic mechanisms of vitamin E regeneration were discovered in liver organelles and platelets [11-13]. In particular, NADPH-driven electron transport in liver microsomes and mitochondria was reported to reduce phenoxyl radicals which provides for the recycling of phenolic antioxidants including vitamin E [11,12,14,15]. Thus it appears that two NADPH-driven reactions may interact in the course of lipid peroxidation in conflicting manners by: (i) activating oxygen and initiating lipid peroxidation; this requires the presence of catalytic concentrations of transition metal complexes, and (ii) reducing phenoxyl radicals of phenolic antioxidants thus bolstering antioxidant protection. This reaction is independent of transition metals. The latter antioxidant effect of NADPH-dependent regeneration of phenolic antioxidants may be masked by rapid irreversible consumption of phenolic antioxidants during transition metal-catalyzed lipid peroxidation.

To elucidate the role of NADPH-dependent phenoxyl radical reduction in the antioxidant protection of membranes, in the present study we compared the effects of NADPH on the accumulation of lipid peroxidation products (TBARS) and vitamin E consumption in two different systems in which peroxidation was initiated by: (i) NADPH+iron - transition metal-dependent system, or (ii) a lipid-soluble azo-initiator of peroxyl radicals, 2,2'-azo-bis(2,4-dimethyl-valeronitrile (AMVN) - transition metal-independent system. In addition, the effects of NADPH on AMVN-induced generation of vitamin E phenoxyl radicals were studied.

MATERIALS AND METHODS

Membrane preparations. The livers of male Wistar rats weighting 220-240 g were perfused with 1.15% ice-cold KCl and homogenized in Potter homogenizer. Microsomal fractions were obtained by differential centrifugation (10 000g x 20 min followed by 100 000g x 60 min). The concentration of membrane protein was determined by Bio-Rad Protein Assay reagent.

Microsomes with decreased endogenous vitamin E concentration animals were isolated from animals fed ad libitum a vitamin E-free diet for 6 weeks [11]. The vitamin E content in microsomes from the vitamin E-deficient and the control groups is shown in Table.

Lipid peroxidation induction and assay. Two systems were used to induce lipid peroxidation in rat liver microsomes: (i) AMVN in the presence of deferoxamine and (ii) NADPH. Incubation medium contained: microsomes 1 mg protein/ml in 0.1 M Phosphate buffer, pH 7.4 at 40°C, NADPH 0.5 mM, or AMVN 5.0 mM, deferoxamine 25 μM. The reaction was started by addition of either NADPH or AMVN. Denaturation of microsomes was performed by a heat treatment (1 min at 90°C) which resulted in a complete inhibition of NADPH oxidation. The concentration of lipid peroxidation products was monitored spectrophotometrically by the formation of 2-thiobarbituric acid reactive substances (TBARS) using the molar extinction at 535 nm, $\varepsilon=1.56 \times 10^5\ M^{-1}\ cm^{-1}$) [16].

HPLC measurements of α-tocopherol content. α-Tocopherol content in microsomes was assayed by reverse phase HPLC using a C-18 column (Waters, Inc.) with an in-line electrochemical detector. The eluent was methanol:ethanol 1:9 (v/v), 20 mM lithium perchlorate. α-Tocopherol was extracted into hexane from sodium dodecyl

sulfate-treated samples as described earlier [17]. Incubation conditions: microsomes 1 mg protein/ml in 0.1 M Phosphate buffer, pH 7.4 at 40°C, NADPH 0.5 mM, or AMVN 5.0 mM, deferoxamine 25 μM.

Generation of chromanoxyl radicals. Phenoxyl (chromanoxyl) radicals were generated from α-tocopherol homologue, chromanol-α-C6 using AMVN. The incubation medium (100 μl) contained: microsomes (35 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4 at 40°C, deferoxamine 25 μM, and AMVN (5.0 mM). The concentration of exogenously added chromanol was 60 nmoles/mg protein The reaction was started by addition of AMVN and was carried out at 40°C.

ESR measurements. ESR measurements were made on a Varian E 109E spectrometer in gas permeable Teflon tubings (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan N.J. USA). The gas permeable tube (approximately 8 cm in length) was filled with 60 μl of a mixed sample, folded into quarters and placed in an open 3.0 mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. Spectra were recorded at 100 mW power, 2.5 gauss modulation at 100 KHz, and 25 gauss/minute scan time at 40°C under aerobic conditions by flowing oxygen through the ESR cavity.

Reagents. NADPH, Tris-HCl, 2-thiobarbituric acid, trichloroacetic acid, sodium phosphates were from Sigma, AMVN from PolySciences, Inc., Warrington, PA. α–Tocopherol homologue - 2,5,7,8-tetramethyl-2(4'-methyl-pentyl)-6-hydroxychromane (chromanol-α-C6) was a gift from Prof. R.P Evstigneeva (Institute of Fine Chemical Technology, Moscow, Russia).

RESULTS AND DISCUSSION

Effects of NADPH on microsomal lipid peroxidation. Incubation of rat liver microsomal suspension in the presence of NADPH was accompanied by a continuous accumulation of lipid peroxidation products (TBARS) (Fig. 1). The rate of TBARS accumulation decreased in the course of incubation. The addition of deferoxamine, a well known iron chelator, resulted in a complete inhibition of lipid peroxidation indicating that the presence of adventitious iron was crucial for NADPH-supported lipid peroxidation. The inhibitory effect of deferoxamine on NADPH-induced lipid peroxidation was reported earlier [18].

AMVN-induced lipid peroxidation in microsomes was linear over time and was not affected by the addition of deferoxamine (Fig. 1). When NADPH was added

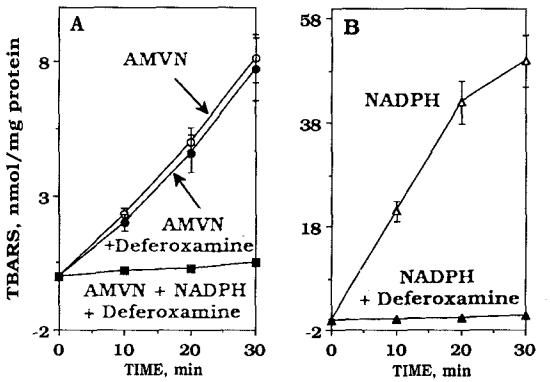


Fig. 1. Accumulation of lipid peroxidation products (TBARS) in rat liver microsomes incubated in the presence of NADPH, AMVN and deferoxamine.
A - AMVN-induced lipid peroxidation; B- NADPH-induced lipid peroxidation.

TABLE 1
Effect of NADPH on AMVN-induced lipid peroxidation in rat liver microsomes with different endogenous vitamin E content

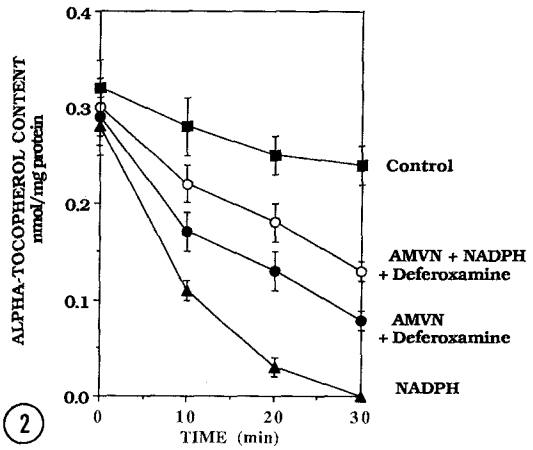
Source of microsomes	vitamin E nmoles/mg protein	Lipid peroxidation microsomes	inhibition, % of control* thermally denatured microsomes
normal	0.33 ± 0.08	93.6 ± 7.4	5.0 ± 3.0
vitamin E defficient	0.12 ± 0.05	61.0 ± 3.8	7.0 ± 5.0

* Control - AMVN- induced lipid peroxidation in the presence of deferoxamine; incubation time -30 min. The data given are mean values ± SD, n=5. Other conditions see in **Materials and** Methods.

to the microsomal suspension incubated in the presence of AMVN and deferoxamine, lipid peroxidation was almost completely suppressed. This data suggests that NADPH-driven electron transport can inhibit lipid peroxidation in liver microsomal membranes when it is not involved in the iron-catalyzed peroxidation. One of the possible mechanisms of this inhibitory action of NADPH on AMVN-induced microsomal lipid peroxidation might be via the enzymic recycling of endogenous vitamin E from its phenoxyl (tocopheroxyl) radicals generated by interaction with peroxyl radicals. To test this hypothesis we compared the effects of NADPH on: (i) AMVN-induced lipid peroxidation in liver microsomes with two different levels of endogenous vitamin E, and (ii) vitamin E consumption by lipid peroxidation.

Similarly to microsomes from the rats fed a control diet, deferoxamine inhibited NADPH-induced lipid peroxidation in vitamin E-deficient microsomes. However in vitamin E-deficient microsomes the inhibitory effect of NADPH on AMVN-induced lipid peroxidation was significantly weaker than in control microsomes (Table I). This indicates that vitamin E is essential for the inhibitory effect of NADPH on AMVN-induced lipid peroxidation. In thermally denatured microsomes NADPH did not inhibit AMVN-induced lipid peroxidation both in control and vitamin E-deficient microsomes (Table I). This suggests that the enzymic recycling of vitamin E may be responsible for the inhibitory effect of NADPH on AMVN-induced lipid peroxidation.

Effects of NADPH on microsomal vitamin E. Incubation of microsomes with NADPH resulted in a rapid depletion of endogenous vitamin E over 30 min (Fig. 2). AMVN in the presence of deferoxamine oxidized almost 2/3 of the endogenous vitamin E pool over a 30 min incubation period. When NADPH was added to the microsomal suspension incubated with AMVN and deferoxamine, a protective effect was observed: vitamin E consumption was decreased. The NADPH-dependent protection of vitamin E consumption against AMVN-induced oxidation was not observed in thermally denatured microsomes. Thus in the absence of iron NADPH does not act as an efficient inducer of lipid peroxidation. Instead it spares vitamin E in microsomal membranes. It is very likely that the NADPH-driven electron transport in microsomes,



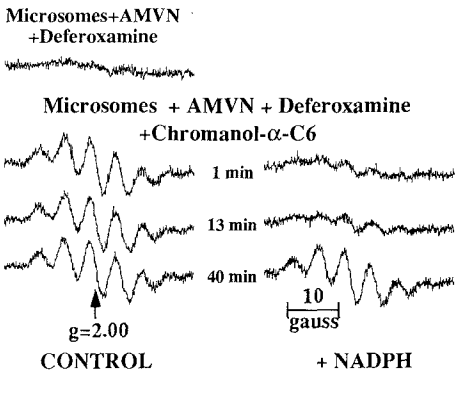


Fig. 2. α-Tocopherol consumption in the course of incubation of rat liver microsomes with NADPH, AMVN and Deferoxamine.

Fig. 3. ESR spectra of the chromanoxyl radicals generated from chromanol-α-C-6 by AMVN in rat liver microsomes in the presence of deferoxamine. Effect of NADPH.

which has been shown to reduce phenoxyl radicals, partly replenishes vitamin E loss during oxidation.

Effects of NADPH on chromanoxyl (phenoxyl) radicals in microsomes. In microsomes with normal or decreased levels of endogenous vitamin E no resolved ESR signal of its phenoxyl (chromanoxyl) radical were detected in the presence of AMVN (Fig. 3). When an exogenous vitamin E homologue with a shorter 6-carbon side chain (chromanol-α-C-6) was added to the microsomal suspension, its oxidation by AMVN in the presence of deferoxamine produced steady-state concentrations of its phenoxyl (chromanoxyl) radicals high enough to be directly detected by ESR (Fig. 3). The steady-state concentration of chromanoxyl radicals did not change significantly over 30 minutes of incubation. Addition of NADPH resulted in a transient disappearance of the chromanoxyl radical ESR signal (Figs. 3,4). The chromanoxyl radical ESR signal reappeared after NADPH was consumed. In an earlier study with the (lipoxygenase+linolenic acid) enzymic oxidation system which generates chromanoxyl radicals, the NADPH-induced transient disappearance and subsequent reappearance of the characteristic ESR signal has been shown to be due to the electron-transport driven reduction of chromanoxyl radicals in rat liver microsomes. In accordance with this previous finding, the present data indicates that NADPH-dependent reduction of AMVN-induced chromanoxyl radicals occurs in microsomes. This regeneration of vitamin E homologue bolsters antioxidant protection and inhibits lipid peroxidation.

The results suggest that in addition to its well known role in initiating lipid peroxidation in the presence of transition metals, NADPH-driven electron transport is able to inhibit lipid peroxidation in liver microsomes possibly via the mechanism of antioxidant recycling. In the presence of exogenously added or adventitious transition

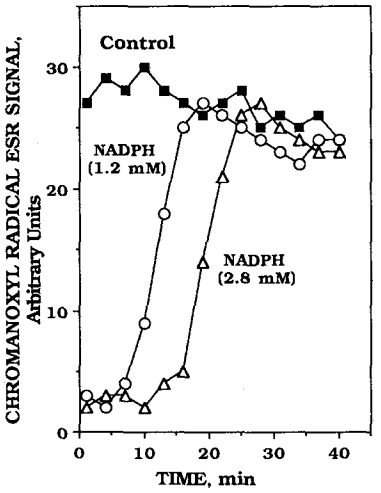


Fig. 4. Effect of NADPH on the time course of AMVN-induced chromanoxyl radicals in rat liver microsomes.

metals this antioxidant function of NADPH-driven electron transport is masked by its high efficiency in oxygen activation resulting in lipid peroxidation (Fig. 5). When lipid peroxidation is induced independent of transition metals (by AMVN in the presence of deferoxamine in the experiments presented) the antioxidant function of NADPH-driven electron transport in inhibiting lipid peroxidation becomes apparent. The availability of transition metals in the form appropriate for oxygen activation

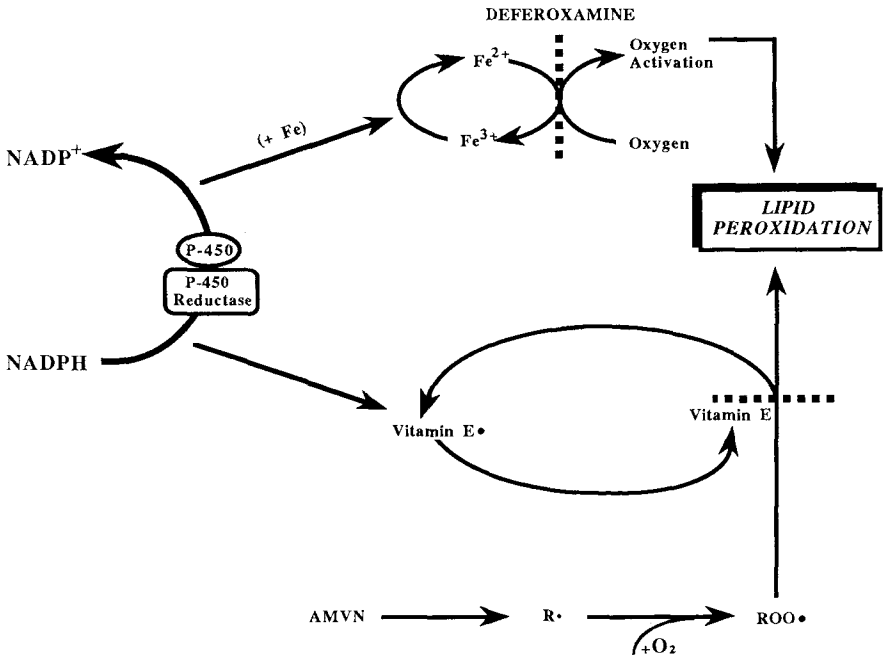


Fig. 5. Scheme illustrating pro- and/or anti-oxidant effects of NADPH on lipid peroxidation in rat liver microsomes.

predetermines pro- and/or anti-oxidant function of NADPH-driven electron transport in microsomes.

A heat-labile glutathione-dependent lipid peroxidation inhibiting factor has been found in liver microsomes which requires vitamin E for its functioning [19,20]. Although the enzymic NADPH-dependent antioxidant mechanism described in this study does not require exogenous glutathione, its activity may be enhanced by dihydrolipoic acid or glutathione in the presence of ascorbate as has been recently reported [21].

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