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Effect of N-Nitrosodiethylamine on Lipid Peroxidation and Antioxidant Enzymes in Rat Liver Mitochondria: Protective Role of Antioxidants

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Since Magee and Barnes (1956) reported the carcinogenicity of nitrosamines in rats, the N-nitroso compounds have been implicated in the aetiology of some human cancers. Nitrosamines have been found in foods such as meat and dairy products, and alcoholic beverages (Song and Hu 1988). In humans the average intake of volatile nitrosamines from food is approximately 1 µg/day (Scanlan 1983). Nitrosamines such as N-nitrosodiethylamine (NDEA) have been suggested to cause oxidative stress and cellular injury and the process may involve free radicals (Bartsch et al. 1989). Free radicals can initiate lipid peroxidation (LPO) which is the oxidative deterioration of membrane lipids which, in turn, can lead to the development of a diseased state (Halliwell et al. 1992).

Mitochondria utilize oxygen at a high rate and have a large content of polyunsaturated fatty acids together with peroxidation catalysts such as iron. Therefore, mitochondria potentially are at risk of LPO induced injuries. The polyunsaturated fatty acids in mitochondria contain two major phospholipids, i.e., phosphatidylchohne and phosphatidylethanolamine, which account for about 80% of the total phospholipids. Cardiolipin, a phosphohpid specific for mitochondria accounts for nearly 18% of the total phospholipids containing approximately 90% unsaturated fatty acids (Daum 1985). The peroxidation of these lipids may take place and can be stimulated by several types of catalysts and by the metabolic products of xenobiotics (Sies 1991). Mitochondria possess antioxidant defence enzymes such as superoxide dismutase (SOD), catalase (CAT), enzymes of the glutathione system and the antioxidants, reduced glutathione (GSH), vitamin C and vitamin E. The antioxidant defence system may be partially or completely overwhelmed under oxidative stress conditions. Therefore, the present investigation was undertaken to examine the effects of NDEA on LPO and the antioxidant defence enzymes in isolated rat liver mitochondria. Antioxidants were also added to mitochondrial incubations in order to establish the involvement of free radicals in NDEA-induced LPO.

MATERIALS AND METHODS

All chemicals were of analytical grade. NDEA, glutathione reductase (GR), cumene hydroperoxide and 1-chloro, 2,4dinitrobenzene (CDNB) were procured from Sigma Chemical Company, St. Louis, Missouri, U.S.A. NDEA was stored in

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a dry, dark place to control the potential photolysis of NDEA which produces the alkylaminyl radical capable of reducing O_2 and generating superoxide ($O_2^{\cdot-}$) anion (Bartsch et al. 1989).

Male albino rats (Wistar strain) weighing 150-180 g were allowed free access to pellet diet (Godrej, India) and water. The animals were fasted overnight before sacrificing. Livers were washed in phosphate buffered saline (PBS), pH 7.4 (1:9, v/v), and blotted with filter paper. A 10% tissue homogenate was prepared in 0.15 M potassium phosphate buffer, pH 7.4 containing 0.25 M sucrose using a motor driven teflon pestle homogeniser. The homogenate was centrifuged at 1500 g for 10 min at 4°C. The supernatant fraction was then collected and centrifuged at 10000 g for 30 min at 4°C and mitochondria were obtained according to the method of Mustafa (1974). Mitochondria were procured from livers of each of the 6 animals and experiments were performed separately on each sample. The mitochondrial fraction was tested for its purity by the reduction of NADP and the increase in absorbance at 340 nm (Sottocasa 1976). Protein content of the mitochondrial fraction was 5.0-6.0 mg/ml (Lowry et al. 1951).

LPO was determined by the thiobarbituric acid (TBA) reaction with malonyldialdehyde (MDA), a product formed due to the peroxidation of lipids, according to the method of Ohkawa et al. (1979). The reaction mixture, in 20 ml screw-capped vials, consisted of 0.2 ml mitochondrial suspension, 0.2 ml 8.1% sodium dodecyl sulfate solution, 1.5 ml 20% acetic acid solution (adjusted to pH 3.5 with 1N NaOH) and 1.5 ml 0.8% TBA solution. Distilled water was added to make up the volume to 4.0 ml. A tissue blank was prepared for each sample by substituting the mitochondrial fraction with distilled water. The reaction mixture was heated in a waterbath at 95°C for 60 min. The vials were allowed to cool at room temperature and 2 ml of the solution was transferred to a centrifuge tube to which an equal volume of 10% TCA was added. The solution was mixed and centrifuged at 5000 g for 5 min. An aliquot of the supernatant was measured spectrophotometrically at 532 nm. LPO was expressed as nmoles of MDA formed/100 mg protein using a molar extinction coefficient of MDA as 1.56×10^5.

SOD activity was determined by the measurement of the ability of the enzyme to inhibit the autoxidation of pyrogallol (Marklund and Marklund 1974). Catalase was assayed by the decomposition of H_2O_2 (Aebi 1983). Glutathione S-transferase (GST) was estimated by conjugation of the sulfhydryl group with CDNB (Habig et al. 1974). GR was assayed by the method described by Carlberg and Mannervik (1985). The activity of total glutathione peroxidase (GSHPx) in mitochondrial suspensions was determined by the method of Paglia and Valentine (1967). The total GSHPx activity was determined using cumene hydroperoxide as substrate and the activity of Se-dependent GSHPx was measured with H_2O_2 as substrate. All enzyme assays were performed in duplicate and in a two-fold concentration range. Statistical analysis was done using Student's t-test. Probability levels of less than 5% were considered to be significant.

RESULTS AND DISCUSSION

Rat liver mitochondria showed a significant increase in LPO when exposed to different concentrations of NDEA for varying time periods (Table 1). The increase in LPO was much higher when the mitochondria were exposed to NDEA for 60 min as compared to exposure times below 30 min. Mitochondria incubated with different concentrations of NDEA for 60 min showed an 4 fold increase in LPO compared to control incubations.

CAT and GR activities, but not SOD activity, in rat liver mitochondria decreased with increasing concentrations of NDEA exposure (Table 2). In contrast, GST activity increased with NDEA exposure. Total GSHPx activity in rat liver mitochondria increased only at 10.0 mM NDEA while Se-GSHPx was decreased at all NDEA concentrations (Table 2). The effect of antioxidants added to the reaction mixture on NDEA-induced oxidative stress is shown in Table 3. Antioxidants (1mM) decreased LPO significantly compared to liver mitochondria treated with NDEA alone.

LPO in rat liver mitochondria increased with increasing NDEA concentrations and exposure time. Increases in LPO may be related to the presence of high amounts of polyunsaturated fatty acids present in mitochondria but may also be due to decreases in GR and CAT activities in mitochondria treated with NDEA. Increased amounts of H_2O_2 may be responsible for the inhibition of Se-GSHPx in rat liver mitochondria incubated with NDEA and for the increased peroxidative damage. The non-significant variation in total GSHPx upto 1.0 mM NDEA exposure suggests that Se-independent GSHPx reduces lipid peroxides.

GSH conjugation by GST is particularly important and can lead to efficient detoxification of hydroperoxides. The increased activity of GST which was observed in response to NDEA intoxication is likely related to increased numbers of toxic electrophiles. These results clearly suggest that NDEA-induced toxicity is mainly due to peroxidative damage which then leads to cellular injury. Previous studies have shown that NADPH in the presence of ferric ions and ADP increases LPO in rat liver microsomes and mitochondria accompanied by swelling and marked loss of mitochondrial function (Morehouse et al. 1984). These results suggest that mitochondrial cytochrome P-450 may have a role in induction of LPO similar to that played by cytochromes in microsomes. $()_2^{-\cdot}$ anions produced by several autoxidizable substances such as quinonoid compounds can be reduced in a one electron process by flavoenzymes, mitochondrial NADP-cytochrome C reductase, leading to the formation of semiquinone. Under aerobic conditions, semiquinone can be reoxidized with the formation of $()_2^{-\cdot}$ and secondarily H_2O_2, hydroxyl radical and singlet oxygen (Bindoli 1988).

Table 1. Effect of NDEA on lipid peroxidation in rat liver mitochondria

NDEA (mM)	Exposure time (min)			
	10	20	30	60
0.01	413±13	440±26	432±28	861±25
0.10	508±27	459±19	447±28	871±25
1.00	578±36	508±34	456±31	926±21
10.00	457±24	460±27	406±20	925±27

Values are mean±S.E.M of 6 experiments and the results are expressed in nmoles of MDA formed/100 mg protein.
LPO value obtained after incubation of mitochondria without NDEA for 60 min was 225±16.
All values were significant at p<0.00l when compared to the 60 min control value without NDEA.

Table 2. Effect of NDEA on antioxidant enzyme activities in rat liver mitochondria

NDEA (mM)	CAT	SOD	GR	GST	GSHPx	Se-GSHPx
0.0	308±6	7.0±0.3	23.8±1.6	177+7	31.4+2.8	22.8±0.3
0.01	211±8^{c}	6.8±0.1	25.9±1.7	227±13^{a}	28.1±1.4	13.1±1.1^{c}
0.10	203±3^{c}	6.9±0.1	17.0±0.3^{b}	198±18	25.3±1.4	12.3±0.8^{c}
1.00	174±3^{c}	6.8±0.3	18.9±0.8^{a}	207±5^{b}	38.5±3.6	11.5±0.7^{c}
10.00	179±5^{c}	6.6±0.3	16.7±1.2^{b}	211±11^{a}	41.3±2.3^{b}	12.7±1.2^{c}

Values are mean+S.E.M. of 6 experiments.
Rat liver mitochondria were incubated with differing concentrations of NDEA for 60 min at 37°C in sucrose/phosphate buffer, pH 7.4.
Units of enzyme actitivies are expressed as: for CAT, µmoles of H_2O_2 decomposed/mg protein/min; for SOD, units/mg protein; for GR, GSHPx and Se-GSHPx, nmoles of NADPH oxidized/mg protein/min; for GST, nmoles of CDNB conjugatedlmg proteinlmin.
p values: ^{a}<0.05; ^{b}<0.01; ^{c}<0.001.

Table 3. Effect of NDEA on lipid peroxidation in rat liver mitochondria in the presence of exogenously added antioxidants

Treatment	Lipid peroxidation
Control	225±16
NDEA	925±27[c]
NDEA+EDTA	419±7[c]
NDEA+sodium benzoate	375±25[c]
NDEA+succinic acid	279±6[a]
NDEA+GSH	260±7
NDEA+ascorbic acid	270±6[a]

Values are mean±S.E.M. of 6 experiments and the results are expressed in nmoles of MDA formed/100 mg protein.
Rat liver mitochondria were incubated with 10 mM NDEA and 1 mM of each antioxidant for 60 min at 37°C in sucrose/phosphate buffer, pH 7.4. NDEA and antioxidants were prepared in 0.15M phosphate buffer pH 7.4.
p values (NDEA plus antioxidants treated versus NDEA alone and NDEA treated versus control): [a]<0.05; [c]<0.001.

NDEA-induced oxidative stress can be controlled by antioxidants which act in aqueous phase as well as in membrane lipids. Previously, respiratory substrates, particularly succinate, have been shown to effectively inhibit NADPH dependent LPO in isolated rat liver mitochondria (Meszaros et al. 1982). Succinate inhibited not only MDA formation but also protected mitochondrial proteins from the consequences of free radical attack. Succinate protected against permeability changes of the membranes proportional to the protection against LPO (Szabados et al. 1989). Succinate protection against NADPH dependent LPO appears to be related to the reduction of coenzyme. In ubiquinone-depleted submitochondrial particles, protection by succinate was completely abolished when LPO was induced by NADPH. However, succinate was found to produce significant inhibition during cumene hydroperoxide induction. Reduction of cytochrome P-450 was suggested as the mechanism to explain the effect of succinate on cumene hydroperoxide dependent LPO, since the reduced enzyme can act as a peroxidase in the cumene hydroperoxide system (Cavallini et al. 1984). Thus, succinate is involved in the protection of mitochondria from free radical attack initiated by various agents.

EDTA in appropriate concentrations inhibits iron-mediated MDA formation. In fact, this chelating agent can either stimulate or inhibit iron salt-stimulated LPO according to the ratio of the concentration of the chelator to that of the iron salt; usually high EDTA/Fe^{2+} ratios inhibit LPO (Gutteridge et al. 1979). In the present study, EDTA at a concentration of 1 mM strongly inhibited NDEA induced LPO in rat liver mitochondria.

Mitochondria contain GSH, GSHPx and GR that catalyze the reduction of $NADP^{+}$ from NADH (Sottocasa 1976). This system contributes to the protection of mitochondria from LPO. Exogenous addition of GSH to NDEA treated rat liver mitochondria probably acted by reducing endogenous iron and peroxides, thus significantly preventing the lipoperoxidative process.

Hydroxyl radicals cause membrane damage by initiating LPO. Sodium benzoate, a hydroxyl radical scavenger, was found to inhibit NDEA-induced LPO in mitochondria. Of the endogenous antioxidants, ascorbic acid has been proposed as the most effective scavenger of free radicals in aqueous environments. Ascorbic acid appears to trap virtually all peroxyl radicals in the aqueous phase before they diffuse into lipids. Ascorbic acid not only completely protects lipids from peroxidative damage but also spares vitamin E, urate and bilirubin. Ascorbic acid belongs to the frst line of antioxidant defences against lipid soluble peroxyl radicals and is as helpful as vitamin E in the prevention and treatment of diseases and degenerative processes caused by oxidant stress (Frei et al. 1989).

In NDEA-induced rat hepatomas impairment of the mitochondrial respiratory chain activity was shown to be due to oxygen free radicals and a general collapse of the antioxidant enzymatic system (Boitier et al. 1995). This oxidation versus antioxidant imbalance in NDEA-induced oxidative stress may be responsible for increased LPO in rat liver mitochondria. In conclusion, since treatment with various antioxidants significantly lowered NDEA-induced LPO in rat liver mitochondria, free radical scavengers may have an important function in reducing NDEA-induced oxidative stress.

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