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INHIBITION OF LIPID PEROXIDATION BY PARAQUAT: SITE OF INHIBITION IN THE CYTOCHROME P-450-DEPENDENT STEROID HYDROXYLASE SYSTEM FROM BOVINE ADRENAL CORTEX MITOCHONDRIA

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Summary. We have found that NADPH-dependent lipid peroxidation in bovine adrenal cortex mitochondria is strongly inhibited by paraquat. The site of the inhibition of the lipid peroxidation by paraquat has been examined. Paraquat neither inhibits NADPH-2,6-dichlorophenolindophenol nor NADPH-cytochrome c reductase activities However, paraquat is able to retard the rate of reduction of cytochrome P-450 by NADPH. The spectrophotometric measurements provide the first evidence that lipid peroxidation in adrenal cortex mitochondria involves cytochrome P-450 and that the inhibitory effect of paraquat on lipid peroxidation is due to reoxidation of reduced cytochrome P-450 by the reagent.

INTRODUCTION

Paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride; methyl viologen) is a widely used herbicide that is toxic (1-4) and highly mutagenic (5). After oral administration, paraquat markedly accumulates in the lung, with indications that kidney, adrenal and liver also have some ability to accumulate paraquat (6).

The biochemical events underlying the toxicity of paraquat are not well understood although one proposed mechanism involves its role in peroxidation of membrane lipids (7). It has been suggested that the lipid peroxidation is mediated through single electron reduction of paraquat, catalyzed by microsomal NADPH-cytochrome c reductase, with the subsequent transfer of the electron from reduced paraquat to molecular oxygen to form superoxide anion (7). Superoxide generated by cyclic reduction-oxidation of paraquat has been demonstrated to stimulate peroxidation of rat liver microsomal lipid (7). However, Kornbrust and Mavis (8) have shown that paraquat inhibited rat liver and lung microsomal peroxidation. Similarly, no stimulation of peroxidation was produced by paraquat in rabbit or human lung microsomes. Further, paraquat enhanced peroxidation in mouse lung and liver microsomes (8). On the other hand, Misra and Gorsky (9) have recently reported inhibition of NADPH-dependent lipid peroxidation in bovine lung microsomes by paraquat.

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Abbreviation: MDA, malondialdehyde.

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It has been shown that NADPH-dependent microsomal lipid peroxidation is catalyzed by NADPH-cytochrome P-450 reductase and it has been suggested that cytochrome P-450 (P-450) is also involved in the reaction (10). Adrenal cortex mitochondria also contain P-450, adrenodoxin (an iron-sulfur protein) and adrenodoxin reductase (an NADPH-dependent diaphorase), all of which are involved in steroid hydroxylation reactions. Adrenocortical mitochondria are very effective in lipid peroxidation (11). However, little is known about the mechanism of this reaction in mitochondria.

Since there exists some controversy in the literature concerning paraquat and lipid peroxidation, the present work was undertaken to study the effects of paraquat on NADPH-dependent lipid peroxidation in adrenal cortex mitochondria. The experimental results presented here show the involvement of P-450 in NADPH-dependent lipid peroxidation and inhibition by paraquat of this process.

MATERIALS AND METHODS

NADPH, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), D-mannitol were purchased from Sigma. EDTA was from Aldrich; methyl viologen (paraquat) from ICN Pharmaceuticals, Inc. Amphenone B, aminoglutethimide, and metyrapone were generous gifts from Ciba.

Fresh bovine adrenal glands were collected at a local slaughterhouse and brought to the laboratory on ice within 1 h after sacrifice. The gland was bisected longitudinally and medulla was removed. The capsule was scraped from the cortex, which was then homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.32 M mannitol and 0.1 mM EDTA with a Teflon homogenizer. The homogenate was centrifugated at 750 x g for 10 min. The pellets were discarded and the resulting supernatant was centrifuged at 1950 x g for 3 min followed by 9750 x g for 10 min. The mitochondria were mechanically separated from any residual material at the bottom of the tube and were washed once in a solution containing 10 mM Tris-HCl buffer (pH 7.4), 0.32 M mannitol and 0.1 mM EDTA at 7700 x g for 10 min and two times by 10 mM phosphate buffer (pH 7.4) containing no EDTA by centrifugation at 10800 x g for 10 min. These washed mitochondria were resuspended in 10 mM phosphate buffer (pH 7.4). The samples were stored at 0°C for 24 h in order to make the mitochondrial membrane permeable to NADPH. Our mitochondria samples are at least 90% pure and evidence indicates that minor contamination by microsomes is not responsible for the observed lipid peroxidation. Lipid peroxidation was determined by the method of thiobarbituric acid (12) using a molar extinction coefficient of 9×10^4 $M^{-1}cm^{-1}$ at 532 nm (13). The amount of TBA-positive material was expressed as a corresponding amount of malondialdehyde (MDA). Butylated hydroxytoluene in ethanol was added to the TBA-trichloroacetic acid-HCl reagent (final concentration of BHT was 0.01%) to prevent nonspecific chromophore formation during the assay procedure. P-450 was determined by bubbling CO in two cuvettes containing 10 mM phosphate buffer (pH 7.4) and 0.8 mg of mitochondrial protein in a total volume of 1 ml with reduction of the sample cuvette by 0.5 mM NADPH or a few grains of sodium dithionite. A difference spectrum was recorded in a spectrophotometer with an end-on photomultiplier and P-450 reduction was calculated using a molar extinction coefficient of 91×10^3 $M^{-1}cm^{-1}$ for the absorptive increment between 448 and 490 nm (14). Protein was determined by the biuret method using bovine serum albumin as a standard (15).

RESULTS AND DISCUSSION

Adrenal cortex mitochondrial lipid peroxidation and inhibition by paraquat.

In our experiments mitochondria were used which were slightly damaged by suspend-

Table I. NADPH-Dependent Lipid Peroxydation in Bovine Adrenal Cortex Mitochondria

Addition	Malondialdehyde formation (nmoles/mg mitochondrial protein)		Difference between 30 min and 0 min
	Time 0 min	Time 30 min	
None	1.6 ± 0.4[a]	6.0 ± 0.5	4.4 (a)
+ Paraquat	1.5 ± 0.3	5.6 ± 0.4	4.1 (b)
NADPH	1.7 ± 0.5	14.0 ± 1.5	12.3 (c)
+ Paraquat	1.6 ± 0.4	6.6 ± 1.1	5.0 (d)

The incubation was carried out at 37°C for 30 min in 2.5 ml medium containing: 2 mg adrenocortical mitochondrial protein and 10 mM phosphate buffer (pH 7.4). Final concentrations of 0.1 mM NADPH and 0.1 mM paraquat were used. [a]Mean ± standard deviation (n = 3 - 5).

ing in 10 mM phosphate buffer at pH 7.4 (hypotonic solution) and storing at 0°C for 24 h. This procedure was performed to promote the entry of NADPH into the mitochondria and to stimulate MDA formation. In a fresh mitochondrial preparation, there is a negligible amount of MDA after incubation with NADPH. The ability of NADPH alone (without addition of exogenous iron) to promote lipid peroxidation in the damaged mitochondria is shown in Table I. The concentration of NADPH used in these experiments (0.1 mM) is that which produces a maximal effect. The small amount of MDA present in the unincubated (zero-time) mitochondria was subtracted from the value after 30 min incubation (Table I). This NADPH-dependent lipid peroxidation could be strongly inhibited by the addition of paraquat while under the same conditions paraquat had no significant effect on lipid peroxidation in the absence of NADPH. In following experiments, zero-time controls and incubations without NADPH were performed with each experiment and percent inhibition by paraquat was calculated as follows: $100 - \left(\frac{d-b}{c-a} \times 100\right)$ = % of inhibition (for a - d refer to Table I). The inhibition increased dramatically with an increase in paraquat concentration (Table II). Approximately 90% inhibition of 100 µM NADPH-supported MDA formation was observed with 100 µM paraquat. In contrast to reports with other systems, no stimulation of lipid peroxidation by paraquat was observed at any concentration tested. Similar results were obtained when an NADPH-generating system (glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase) was used instead of NADPH. Our results are in accord with Ilett et al (16) and Misra and Gorsky (9), who reported that in the absence of exogenous inorganic iron, a decreased rate of microsomal lipid peroxidation occurred in the presence of paraquat. In a separate preliminary study we have shown that addition of 0.2 mM Fe^{2+} to reaction mixtures increased the lipid peroxidation of bovine adrenal cortex mitochondria about 5-fold, and paraquat at 0.1 mM inhibited the MDA produc-

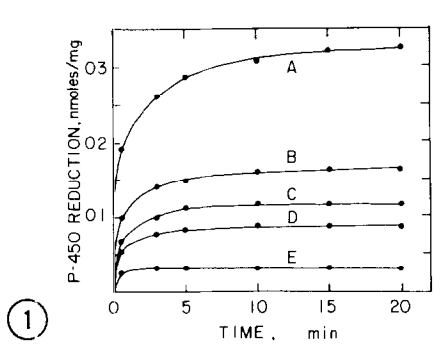
Table II. Inhibition of NADPH-Dependent Lipid Peroxydation in Bovine Adrenal Cortex Mitochondria by Paraquat

Concentrations of Paraquat (μM)	NADPH / Paraquat (mol/mol)	Malondialdehyde formation (nmoles/mg mitochondrial protein)	% of inhibition
None (control)	—	7.2	—
2.5	100:2.5	4.9	32
5	100:5	4.4	39
10	100:10	3.2	55
25	100:25	2.5	65
50	100:50	1.7	76
100	100:100	0.7	90

The experimental conditions were the same as that described in Table I. Final concentratio 0.1 mM NADPH was used (control = 0.1 mM NADPH alone). Various concentrations of paraquat were added to the reaction mixture as indicated in the table.

tion by 20% of the control rate. These results also agree with Misra and Gorsky (9), who observed that paraquat inhibited by 10-18% the NADPH-dependent lipid peroxidation bovine lung microsomes in the presence of Fe^{3+} and ADP.

Inhibition of NADPH-dependent cytochrome P-450 reduction by paraquat. Earlier reports have demonstrated reduction of paraquat by microsomal NADPH-cytochrome c reductase in the presence of NADPH (7). In order to examine the inhibitory effect of paraquat we have studied the action of paraquat on NADPH-2,6-dichlorophenolindophenol (DCPIP) reductase, NADPH-cytochrome c reductase, and P-450 reduction. It was found that paraquat (NADPH:paraquat ratio = 100: 100; mol/mol) had no effect on NADPH-dependent reduction of DCPIP and on NADPH-cytochrome c reductase activities in bovine adrenal cortex mitochondria. However, we found that paraquat strongly inhibits NADPH-dependent reduction of P-450. Figure 1 shows time-dependent reduction of P-450 with and without addition of various concentrations of paraquat. The reduction of P-450 in the presence of NADPH showed two phases. A fast reduction within 5 min and slow rate of reduction after 5 min were observed. Compared with dithionite reduction of P-450, it was found under these conditions that about 33% of total P-450 is reduced by NADPH. As shown in Figure 1, even low concentration of paraquat provided significant inhibition of P-450 reduction. The highest concentrations of paraquat (NADPH: paraquat ratio = 100:20; mol/mol) almost completely inhibited the reduction of P-450. Significantly, at an NADPH to paraquat ratio of 100:25, lipid peroxidation is also strongly inhibited (Table I).



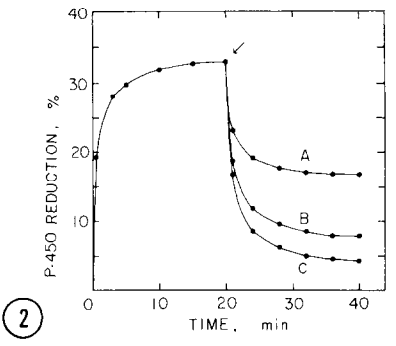


Figure 1. Effects of various concentrations of paraquat on NADPH-dependent reduction of P-450 in adrenocortical mitochondria. The sample cuvette contained 0.8 mg mitochondrial protein (containing 0.81 nmol of P-450), 10 mM of phosphate buffer (pH 7.4), 0.5 mM NADPH and various concentrations of paraquat. Concentrations of paraquat were: A, 0 (control); B, 12.5 μM (NADPH:paraquat = 100:2.5); C, 25 μM (NADPH:paraquat = 100:5); D, 50 μM (NADPH:paraquat = 100:10); E, 100 μM (NADPH:paraquat = 100:20).

Figure 2. Time-dependent effects of paraquat on reduced P-450. The sample cuvette contained 0.8 mg mitochondrial protein (containing 0.81 nmol of cytochrome P-450), 10 mM of phosphate buffer (pH 7.4) and 0.5 mM NADPH. The addition of various concentrations of paraquat was done at the time indicated by the arrow. Concentrations of paraquat were: A, 12.5 μM (NADPH:paraquat = 100:2.5); B, 25 μM (NADPH:paraquat = 100:5); C, 50 μM (NADPH:paraquat = 100:10).

Reoxidation of reduced cytochrome P-450 by paraquat. A lack of an inhibitory effect of paraquat on NADPH-cytochrome c reductase suggested that paraquat would divert electrons from reduced P-450. Consequently we studied the effect of paraquat on the NADPH-reduced P-450. Figure 2 shows that as the concentrations of paraquat increased in the reaction mixture, the amount of reduced P-450 was decreased. We also found that dithionite-reducible P-450 is not destroyed by paraquat. One mg of mitochondrial protein contains 1.01 ± 0.06 nmol of P-450 before and after paraquat addition. These data suggest that P-450 is involved in lipid peroxidation and that the inhibitory effect of paraquat on lipid peroxidation is due to reoxidation of reduced P-450 by the reagent.

Inhibition of lipid peroxidation by steroid hydroxylase inhibitors. To test the above proposal, the effect of inhibitors of P-450-mediated steroid hydroxylation reactions was investigated as follows. Amphenone B, aminoglutethimide and metyrapone are known to be potent inhibitors for adrenocortical P-450-mediated reactions. The mechanism of inhibition is believed to be an interaction of inhibitor with P-450 (17). It was found that these inhibitors decreased MDA production (Table III). The order of potency was: amphenone B > aminoglutethimide > metyrapone. It has been demonstrated that lipid peroxidation could be catalyzed by purified hepatic P-450, thus prompting the suggestion that P-450 may act in a similar fashion during the peroxidation of lipids within intact microsomes (10). Recently, Baird (18) showed that pretreatment of rats with 2-allyl-2-isopropylacetamide, which destroys hepatic microsomal P-450, had no effect upon subsequent

Table III. Effect of Steroid Hydroxylase Inhibitors on Lipid Peroxydation in Adrenal Cortex Mitochondria

Addition	Malondialdehyde formation (nmoles/mg mitochondrial protein)	% of inhibition
NADPH	8.4 ± 0.3[a]	—
+ Amphenone B	0.0 ± 0.0	100
+ Aminoglutethimide	4.0 ± 0.4	52
+ Metyrapone	6.2 ± 0.4	26

The experimental conditions were the same as that described in Table I. Percent of inhibition by steroid hydroxylase inhibitors calculated in the same way as for paraquat. Final concentration of 0.1 mM NADPH, 1 mM amphenone B, 1 mM aminoglutethimide and 1 mM metyrapone were used. [a]Mean ± standard deviation (n = 3 - 4).

NADPH-driven lipid peroxidation in liver microsomes. He also showed that induction of specific forms of P-450 in hepatic microsomes has no effect upon lipid peroxidation in those microsomes. These findings suggested that P-450 may not have a catalytic role in the peroxidation of lipid in intact microsomes. However, our results demonstrate that in bovine adrenal cortex mitochondria P-450 is involved in the NADPH-dependent lipid peroxidation. Further, our studies suggest that paraquat inhibits lipid peroxidation by reoxidation of reduced cytochrome P-450. However, in view of oxidation-reduction potentials of $P\text{-}450_{scc}$ (E'_o = approximately -305 mV for the high spin form and -412 mV for the low spin form) (19) and paraquat (E'_o = -441 mV) (20) the reduction of paraquat by reduced P-450 would occur under certain conditions.

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