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THE RELATIONSHIP BETWEEN NADPH-DEPENDENT LIPID PEROXIDATION AND DEGRADATION OF CYTOCHROME P-450 IN ADRENAL CORTEX MITOCHONDRIA

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Summary. The relationship between NADPH-dependent lipid peroxidation and the degradation of cytochrome P-450 has been studied in bovine adrenal cortex mitochondria. Malondialdehyde formation is accompanied by a corresponding decrease in total cytochrome P-450 content. Inhibitors of lipid peroxidation also prevent the loss of cytochrome P-450, further demonstrating a direct relationship between NADPH-dependent lipid peroxidation and degradation of P-450. To differentiate between cytochrome $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$, steroid-induced difference spectra were used to evaluate P-450 degradation. These measurements provide the first evidence that both P-450's are degraded during NADPH-dependent lipid peroxidation with $P\text{-}450_{11\beta}$ being much more susceptible to this process.

Lipid peroxidation of unsaturated lipids has been studied in a variety of biological systems (1) and, since first reported (2,3), has been associated with the oxidative destruction of membrane lipids. Lipid peroxidation has also been shown to result in inactivation of microsomal and mitochondrial enzymes as well as cytochrome (4,5). Previously, in rat liver microsomes, Levin et al. (6) showed a direct relationship between the loss of P-450 heme groups, the loss of microsomal polyunsaturated fatty acids and the formation of malondialdehyde, the end product of lipid peroxidation. It has also been observed that ferrous ion mediates P-450 degradation and malondialdehyde formation in adrenal cortex mitochondria (7). Recently we have shown that NADPH-dependent lipid peroxidation in adrenal cortex mitochondria involves P-450 (8). Therefore, it was of interest to study whether P-450 is destroyed during this enzymatic process. Because adrenal cortex mitochondria contain at least two separate P-450's, one for 11β-hydroxylation of deoxycorticosterone ($P\text{-}450_{11\beta}$) and the other for cholesterol side-chain cleavage ($P\text{-}450_{scc}$) (9,10), we decided to study the relationship between NADPH-dependent lipid peroxidation and the degradation of both P-450's.

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Abbreviation: MDA, malondialdehyde; $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$, adrenal mitochondrial P-450 cytochromes which function in steroid 11β-hydroxylation and cholesterol side-chain cleavage, respectively.

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MATERIALS AND METHODS

NADPH, deoxycorticosterone, 20 α-hydroxycholesterol, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), superoxide dismutase were purchased from Sigma Chemical Co. EDTA was obtained from Aldrich Chemical Co. Amphenone B was a generous gift from Ciba.

Preparation of mitochondria from bovine adrenal cortex was performed as described previously (8). The mitochondria were stored at 0°C for 24 h in order to make the mitochondrial membrane permeable to NADPH. Cytochrome P-450 was determined as reported previously (8) using a molar extinction coefficient of 9.1 x 10^4 M^{-1} cm^{-1} for the difference in absorbance between 448 and 490 nm (11). The deoxycorticosterone-induced type I absorbance changes and 20 α-hydroxycholesterol-induced type II absorbance changes were determined at room temperature under aerobic conditions (12). Lipid peroxidation was measured by the formation of the TBA-reactive material as malondialdehyde (MDA) (8). Protein was determined by the biuret method (13) in 0.25% Na deoxycholate with bovine serum albumin as standard. Optical spectroscopy was carried out using a Cary spectrophotometer (model 118) with an end-on photomultiplier.

RESULTS AND DISCUSSION

Figure 1 shows the relationship between P-450 content and malondialdehyde formation. The small amount of MDA present in unincubated mitochondria was

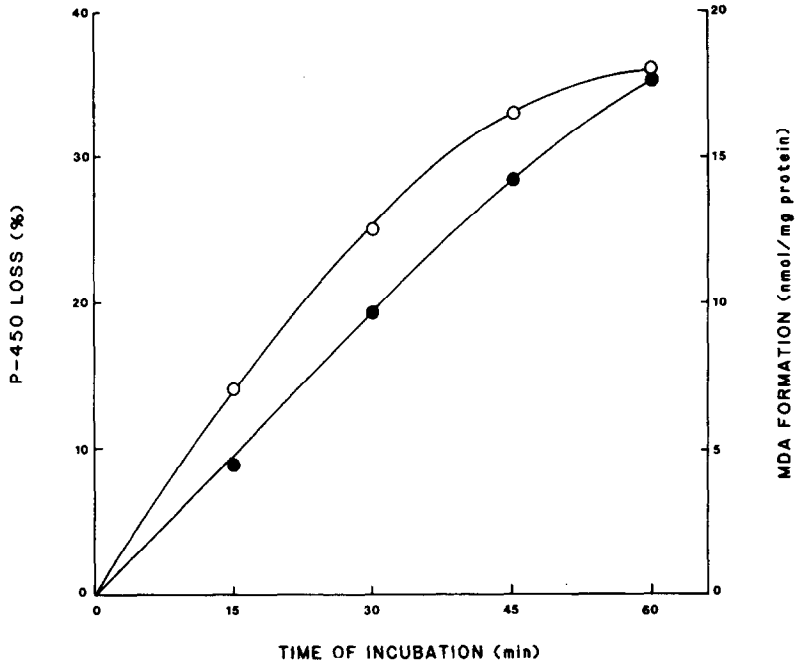

Figure 1. The time-dependent relationship between MDA formation and P-450 content. The incubation was carried out at 37°C under aerobic conditions with constant shaking for 1 h using 20 ml of 10 mM phosphate buffer (pH 7.4) containing 32 mg adrenal cortex mitochondria. A final concentration of 0.2 mM NADPH was used. After incubation, the lipid peroxidation was terminated by adding 1 mM EDTA. 0.5 ml of the samples was taken for MDA determination. All samples were centrifuged at 29000 x g for 15 min. The mitochondria were then washed once in a medium of 10 mM phosphate buffer (pH 7.4) and 1 mM EDTA. The washed mitochondria were resuspended in 10 mM phosphate buffer and used for P-450 content determination.

Table I. The Effect of NADPH-Dependent Lipid Peroxidation on the P-450 Content and on the Steroid-Induced Difference Spectra in Bovine Adrenal Cortex Mitochondria

Sample	MDA content (nmol/mg protein)	P-450 content (nmol/mg protein)	% loss[a] total P-450	Deoxycorticosterone A (388-418) x 10^2	% loss[b] $P\text{-}450_{11\beta}$	20α-OH-Cholesterol A (418-388) x 10^2	% loss[b] $P\text{-}450_{scc}$
Mitochondria unincubated (control)	2.0	1.43	—	4.1	—	6.8	—
Mitochondria incubated with NADPH	21.4	0.97	32	0.35	91	5.2	24
Mitochondria incubated with NADPH and EDTA	1.9	1.27	11	3.4	17	6.5	4

[a]Percent degradation of total P-450 measured by changes in dithionite reducible P-450. [b]Percent degradation of $P\text{-}450_{11\beta}$ or $P\text{-}450_{scc}$ measured by changes in steroid-induced difference spectra. The incubation was carried out at 37°C for 1 h in 40 ml of 10 mM phosphate buffer (pH 7.4) containing 64 mg adrenal cortex mitochondria. Final concentrations of 0.2 mM NADPH and 1 mM EDTA were used. Other experimental procedures were the same as those described in Fig. 1-3.

subtracted from total MDA observed in incubated mitochondria. The net production of MDA, expressed as nmol per mg mitochondrial protein, is shown as a function of time of incubation. MDA production which has been shown to be proportional to polyunsaturated fatty acid disappearance during lipid peroxidation (14,15) parallels the loss of P-450 in adrenal cortex mitochondria.

It is known that deoxycorticosterone (substrate for 11β-hydroxylation) and 20α-hydroxycholesterol (substrate for cholesterol side-chain cleavage) bind to $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$, respectively (12,16). Deoxycorticosterone produces a characteristic type I difference spectrum while 20α-hydroxycholesterol produces a type II difference spectrum (17). In the following experiments we studied the effects of NADPH-dependent lipid peroxidation on total P-450 content and on steroid-induced spectra in adrenal cortex mitochondria. As noted above, dithionite-reducible P-450 was diminished in mitochondria that had undergone lipid peroxidation. Typically, MDA formation of 22.4 ± 1.8 nmol per mg mitochondrial protein was accompanied by a total P-450 loss of 31 ± 2.1 % (Table I). Measurements of steroid-induced difference spectra (Fig. 2 and 3) showed that under these conditions 90 ± 1.7% of $P\text{-}450_{11\beta}$ was destroyed while only 24 ± 1.5% of $P\text{-}450_{scc}$ was lost (Table I). Addition of EDTA, a known inhibitor of lipid peroxidation (14,18), significantly decreased the degradation of the P-450's.

These data demonstrate a direct relationship between NADPH-dependent lipid peroxidation and breakdown of cytochrome P-450 and suggest that $P\text{-}450_{11\beta}$ is more susceptible to this process. On the basis of the different extractability of $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ from the inner membrane, it has been proposed that the states of their association with the inner membrane may differ (9,10, 19). The $P\text{-}450_{scc}$ appeared to be more loosely bound to the inner membrane than $P\text{-}450_{11\beta}$. These facts suggested possible differences in the hydrophobic nature of the two cytochromes. Our present results suggest that changes in $P\text{-}450_{11\beta}$ content may be closely related to peroxidative damage to the structure of the mitochondrial membrane.

It has been reported that adrenal $P\text{-}450_{11\beta}$ is unstable, undergoing spontaneous decomposition during incubation at 37°C (20). Also, it is known that the substrate, deoxycorticosterone, stabilizes $P\text{-}450_{11\beta}$ (but not $P\text{-}450_{scc}$) either by protecting a labile moiety or by transforming the hemoprotein into a more stable conformation (9,20). We have previously shown that steroid hydroxylase inhibitors significantly decrease NADPH-dependent lipid peroxidation (8). Amphenone B was found to be the most effective in inhibiting this process (8). Amphenone B also inhibits both 11β-hydroxylation and cholesterol side-chain cleavage (21). The mechanism of this inhibition is thought to involve an interaction of the inhibitor with P-450 (22). We have, therefore, investigated the effects of amphenone B as well as deoxycorticosterone on

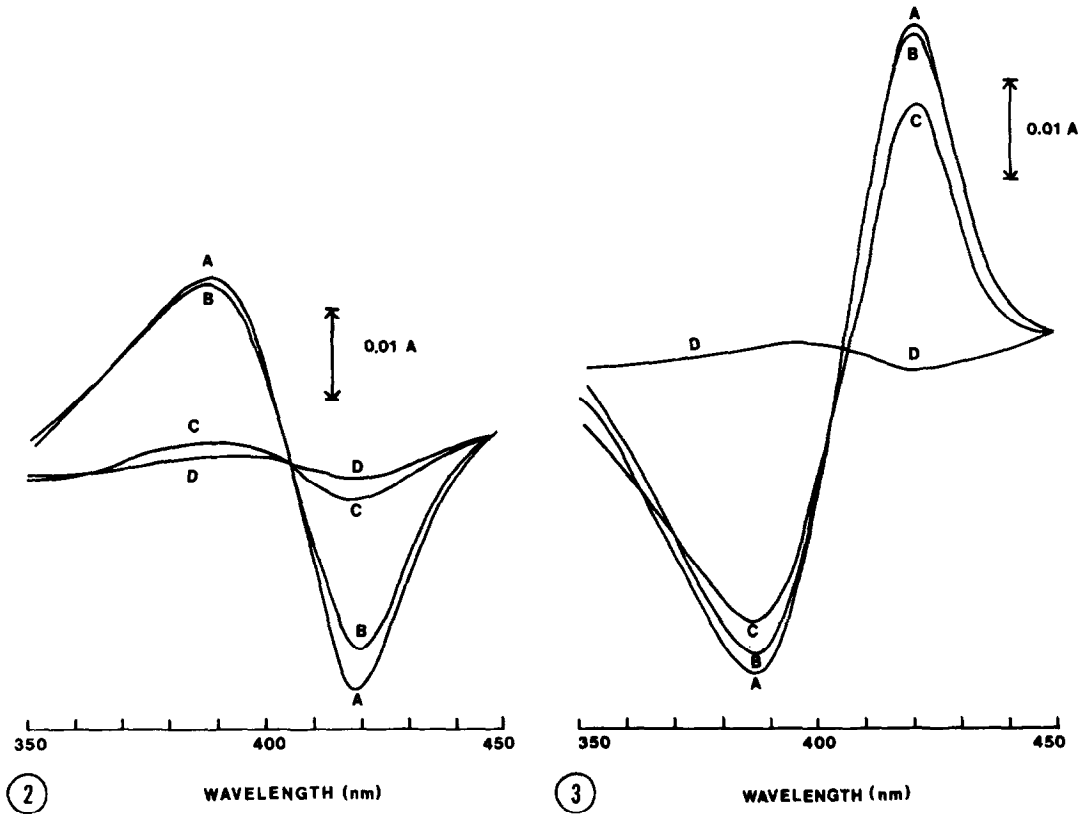

Figure 2. The effect of NADPH-dependent lipid peroxidation on the deoxycorticosterone-induced type I difference spectra of adrenal cortex mitochondria. The reaction mixture contained 1 mg/ml of mitochondrial protein in 10 mM phosphate buffer (pH 7.4) and 1 mM EDTA. The final concentration of deoxycorticosterone was 15 μM. Curve A, unincubated mitochondria (control); Curve B, mitochondria which were incubated in the presence of 0.2 mM NADPH and 1 mM EDTA; Curve C, mitochondria which were incubated in the presence of 0.2 mM NADPH alone; Curve D, mitochondria without deoxycorticosterone addition (baseline).

Figure 3. The effect of NADPH-dependent lipid peroxidation on the 20α-hydroxycholesterol-induced type II difference spectra of adrenal cortex mitochondria. The reaction mixture contained 1 mg/ml of mitochondria protein in 10 mM phosphate buffer (pH 7.4) and 1 mM EDTA. The final concentration of 20α-hydroxycholesterol was 15 μM. Curve A, unincubated mitochondria (control); Curve B, mitochondria which were incubated in the presence of 0.2 mM NADPH and 1 mM EDTA; Curve C, mitochondria which were incubated in the presence of 0.2 mM NADPH alone; Curve D, mitochondria without 20α-hydroxycholesterol addition (baseline).

P-450 degradation. As presented in Table II, deoxycorticosterone had no effect on NADPH-dependent lipid peroxidation while partially protecting $P\text{-}450_{11\beta}$ from degradation. These results are in accord with the above-mentioned observations that deoxycorticosterone binds to $P\text{-}450_{11\beta}$ (but not to $P\text{-}450_{scc}$) (9,20), thereby preventing its degradation. On the other hand, amphenone B which strongly decreases MDA formation completely prevented degradation of both P-450's.

Comparison of the effects of EDTA (Table I) and amphenone B (Table II), both inhibitors of NADPH-dependent lipid peroxidation, suggests that different mechanisms are operative in their protection of P-450. It has recently been

Table II. The Effect of Deoxycorticosterone and Amphenone B on NADPH-Dependent Lipid Peroxidation, P-450 Content and Steroid-Induced Difference Spectra in Bovine Adrenal Cortex Mitochondria

Sample	MDA content (nmol/mg protein)	P-450 content (nmol/mg protein)	% loss[a] total P-450	Deoxycorticosterone A (388-418) x 10^2	% loss[b] $P\text{-}450_{11\beta}$	20α-OH-Cholesterol A (418-388) x 10^2	% loss[b] $P\text{-}450_{scc}$
Mitochondria unincubated (control)	2.1	1.25	–	3.7	–	5.9	–
Mitochondria incubated with NADPH	23.3	0.84	33	0.29	92	4.4	25
Mitochondria incubated with NADPH and deoxycorticosterone	23.0	1.05	16	2.4	35	4.2	29
Mitochondria incubated with NADPH and amphenone B	3.4	1.26	0	3.8	0	6.1	0

[a]Percent degradation of total P-450 measured by changes in dithionite reducible P-450. [b]Percent degradation of $P\text{-}450_{11\beta}$ and $P\text{-}450_{scc}$ measured by changes in steroid-induced difference spectra. The experimental conditions were the same as those described in Table I except that the mitochondria were washed in 10 mM phosphate buffer (pH 7.4) containing 1 mM EDTA followed by centrifugation at 29000 x g for 15 min. This procedure was performed to remove deoxycorticosterone and amphenone B from mitochondria after incubation. Concentrations of 0.2 mM NADPH, 0.1 mM deoxycorticosterone and 0.5 mM amphenone B were used.

reported (18) that EDTA inhibits microsomal NADPH-dependent lipid peroxidation by chelating available iron. Thus we assume that in our experiments EDTA also inhibits the reaction by chelating iron. Although no additional iron was added to the reaction mixture, some iron would always remain in the mitochondrial preparation. On the other hand, amphenone B, which is a low spin inducer of $P\text{-}450_{scc}$ (22) that prevents the further reaction of the reduced form with O_2, inhibits NADPH-dependent lipid peroxidation by binding with P-450. This binding can also protect both P-450's from the small spontaneous degradation that occurs even in the absence of lipid peroxidation. The protection of P-450 by amphenone B with parallel inhibition of MDA formation is in agreement with our recent suggestion (8) that P-450 is involved in enzymatic NADPH-dependent lipid peroxidation in bovine adrenal mitochondria.

Several investigators have proposed the participation of free radicals in NADPH-dependent lipid peroxidation (23-25). Therefore, in a preliminary study we have examined the effects of butylated hydroxytoluene (antioxidant), superoxide dismutase (superoxide scavanger), and ethanol (hydroxyl radical scavenger) on the NADPH-dependent lipid peroxidation in bovine adrenal cortex mitochondria. The addition of butylated hydroxytoluene (1 mM) completely inhibits of MDA formation. However, this reaction is insensitive to superoxide dismutase (150 units) and ethanol (50 mM) indicating that neither superoxide nor hydroxyl radical participate in the NADPH-dependent lipid peroxidation. The inhibitory effect of butylated hydroxytoluene suggests the involvement of free radicals in NADPH-dependent lipid peroxidation in adrenal cortex mitochondria. The ability of microsomal P-450 to generate free radical species from molecular oxygen has been reported (26). Thus, it is possible that free radicals generated by adrenal P-450 could initiate peroxidation of lipids with subsequent degradation of adrenal P-450's (mainly $P\text{-}450_{11\beta}$). However, further studies will be required in order to determine the mechanism of this degradation. Finally, we wish to emphasize the physiological significance of differential degradation of $P\text{-}450_{11\beta}$ upon lipid peroxidation in the adrenal cortex. The cholesterol side chain cleavage reaction would be slightly affected by lipid peroxidation. However, 11β and 18-hydroxylation which are catalyzed by the same $P\text{-}450_{11\beta}$, could be significantly damaged by the peroxidative reaction. In turn, the cortisol and androgen biosynthesis will be inhibited no matter how ACTH stimulation supplies pregnenolone in adrenal cortex.

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