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FACTORS AFFECTING LIPID PEROXIDATION IN GUINEA-PIG ADRENAL MICROSOMES

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Summary

Studies were carried out to examine the effects of and interactions between NADPH, Fe^{2+}, Fe^{3+} and ascorbate on lipid peroxidation in guinea-pig adrenal microsomes. Fe^{2+}, at levels between 10^{-6} and 10^{-3} M, produced concentration-dependent increases in lipid peroxidation in adrenal microsomes; Fe^{2+} had a far greater effect than Fe^{3+}. In liver microsomes, by contrast, Fe^{2+} and Fe^{3+} had quantitatively similar effects on lipid peroxidation. NADPH alone had no effect on malonaldehyde production by adrenal microsomes. However, in the presence of low Fe^{2+} concentrations (10^{-6} M), NADPH stimulated malonaldehyde production; the stimulation was not demonstrable in microsomes which had been heat-treated to inactive microsomal enzymes. In the presence of high Fe^{2+} levels (10^{-3} M), NADPH produced a concentration-dependent inhibition of lipid peroxidation; the inhibition was fully demonstrable in heat-treated microsomes. In the presence of Fe^{3+} (10^{-6} to 10^{-3} M), NADPH had little effect on lipid peroxidation, suggesting that NADPH does not significantly promote the reduction of Fe^{3+} to Fe^{2+} in adrenal microsomes. Ascorbate alone increased malonaldehyde production by adrenal microsomes; maximum stimulation occurred at a concentration of 10^{-4} M. Ascorbate-induced lipid peroxidation was also inhibited by NADPH. Ascorbate ($5 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$ M) synergistically interacted with low levels (10^{-6} M) of Fe^{2+} to enhance malonaldehyde production by adrenal microsomes. The synergism was not demonstrable at high concentrations (10^{-3} M) of Fe^{2+}. At all concentrations (10^{-6} to 10^{-3} M) of Fe^{3+} studied, ascorbate synergistically increased the production of malonaldehyde.

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The results indicate that interactions between various endogenous substances may be important in the control of adrenal microsomal lipid peroxidation and that there are differences in the regulation of adrenal and hepatic lipid peroxidation.

Introduction

Lipid peroxidation, the oxidative deterioration of unsaturated fatty acids, has been demonstrated in vitro in a variety of tissues including the liver, lung, brain, spleen, adrenal, and testis [1—3]. The process of lipid peroxidation has been implicated in the pathogenesis of some of the lesions associated with C_2H_5OH and CCl_4 poisoning [4,5], radiation exposure [6] and exposure to ozone [7], NO_2 [8] and other air pollutants [9]. Some investigators have suggested that lipid peroxidation may also be involved in normal physiological processes such as aging [10]. In addition, lipid peroxidation has been associated with decreased hepatic microsomal drug metabolizing activity and cytochrome *P*-450 levels [11—13].

Because of the possible deleterious effects of lipid peroxidation, much work has gone into the identification of endogenous and exogenous factors that may influence this process in various biological systems. The factors affecting lipid peroxidation have been studied most extensively in the microsomal fraction of liver cells. One of the most potent stimuli of lipid peroxidation in rat liver microsomes is NADPH [14]. Since the microsomal enzyme, NADPH-cytochrome *c* reductase is required for NADPH-induced lipid peroxidation, the process is sometimes referred to as enzymatic lipid peroxidation. Other substances such as Fe^{2+} or Fe^{3+} and ascorbate [15] initiate microsomal lipid peroxidation even after heat-inactivation of microsomal enzymes and, therefore, produce a non-enzymatic type of lipid peroxidation.

One of the tissues in which both enzymatic and non-enzymatic lipid peroxidation has been shown to occur is the adrenal cortex [16,17]. Increases in the rate of lipid peroxidation in adrenal microsomes and mitochondria are associated with decreases in the activities of adrenal cytochrome *P*-450-dependent steroid hydroxylases [16,18]. Thus, lipid peroxidation appears to compromise the activities of steroidogenic enzymes in the adrenal cortex. Although lipid peroxidation may be an important process in the adrenal, relatively little is known about the factors affecting this process. The studies presented in this communication were carried out to examine the actions of and interactions between various endogenous substances (NADPH, Fe^{2+}, Fe^{3+} and ascorbate) on adrenal microsomal lipid peroxidation in the guinea-pig. In addition, effects of these substances on microsomal lipid peroxidation in the adrenal and liver were compared to determine if lipid peroxidation is similarly controlled in the two tissues. A preliminary report of these findings has appeared previously [19].

Methods

Male English Short-Hair guinea-pigs weighing approx. 1000 g were obtained from Camm Research Institute, Wayne, NJ. Animals were maintained under

standardized conditions of light (6.00 a.m.—6.00 p.m.) and temperature (22°C) on a diet of Wayne Guinea-Pig Diet and water ad lib. Guinea-pigs were killed by decapitation between 8.00 and 9.00 a.m. Adrenals and livers were quickly removed and placed in cold 1.15% KCl/0.05 M Tris-HCl buffer (pH 7.4) on ice. Tissues were trimmed free of connective tissue, weighed and homogenized in KCl-Tris buffer. Microsomes were obtained by differential centrifugation as previously described [20] and resuspended in 1.15% KCl/0.05 M Tris buffer (pH 7.4). In some experiments, microsomal enzymes were inactivated by heating the microsomal suspension at 70°C for 2 min. Enzyme inactivation was confirmed by the absence of detectable NADPH-cytochrome *c* reductase, ethylmorphine demethylase and aniline hydroxylase activities [21—23].

Microsomal suspensions were incubated in 25-ml Erlenmeyer flasks in a Dubnoff metabolic incubator at 37°C for 60 min under air. Total volume in each flask was 2.5 ml. Adrenal microsomes were incubated at a concentration of approx. 0.25 mg protein/ml and liver microsomes at approx. 0.75 mg protein/ml. The protein concentrations employed were found to be optimal for lipid peroxidation in each tissue. As indicated, the following agents were added to the reaction flasks prior to incubation: $FeCl_3$ (Fisher Scientific Company, Fairlawn, NJ), $FeSO_4$, L-ascorbic acid and NADPH (Sigma Chemical Company, St. Louis, MO). All of the effects of Fe^{2+} presented in this report were obtained using $FeSO_4$ as the source of Fe^{2+}. However, essentially identical results have also been obtained with $FeCl_2$. Malonaldehyde production, as measured by the thiobarbituric acid test, served as an index of lipid peroxidation. Malonaldehyde was measured according to the method of Ottolenghi [24] as modified by Hunter et al. [25]. Following incubation, 2.0 ml aliquots from each flask were transferred to centrifuge tubes containing 0.5 ml 40% trichloroacetic acid and 0.25 ml 5 N HCl. After mixing, 0.5 ml 2% thiobarbituric acid solution was added and the samples were incubated for 20 min at 90°C. Following incubation, the samples were cooled in an ice bath for 5 min and centrifuged at 30 000 $\times g$ for 5 min in a Sorvall model SS-3 centrifuge. The amount of malonaldehyde in each sample was determined by measuring the absorbance of the supernatants at 532 nm with a Gilford model 300-N spectrophotometer and using a molar extinction coefficient of $1.56 \cdot 10^5\ M^{-1} \cdot cm^{-1}$ [26].

Results

Addition of ascorbate or Fe^{2+} to guinea-pig adrenal microsomes stimulated lipid peroxidation (Fig. 1). Malonaldehyde production was dependent upon the concentration of Fe^{2+} or ascorbate present in the reaction mixture. Ascorbate stimulation of lipid peroxidation was maximal at a concentration of $1 \cdot 10^{-4}$ M and quickly dropped off at higher or lower concentrations. Malonaldehyde production increased with increasing amounts of Fe^{2+} up to a concentration of at least $1 \cdot 10^{-3}$ M. Higher concentrations of Fe^{2+} were not studied because of solubility limitations in the reaction mixture. The stimulatory effects of Fe^{2+} and of ascorbate on adrenal microsomal lipid peroxidation were fully demonstrable in heat-treated microsomes (data not shown), indicating the non-enzymatic nature of the stimulation by each.

The time courses for Fe^{2+}- and ascorbate-induced lipid peroxidation are

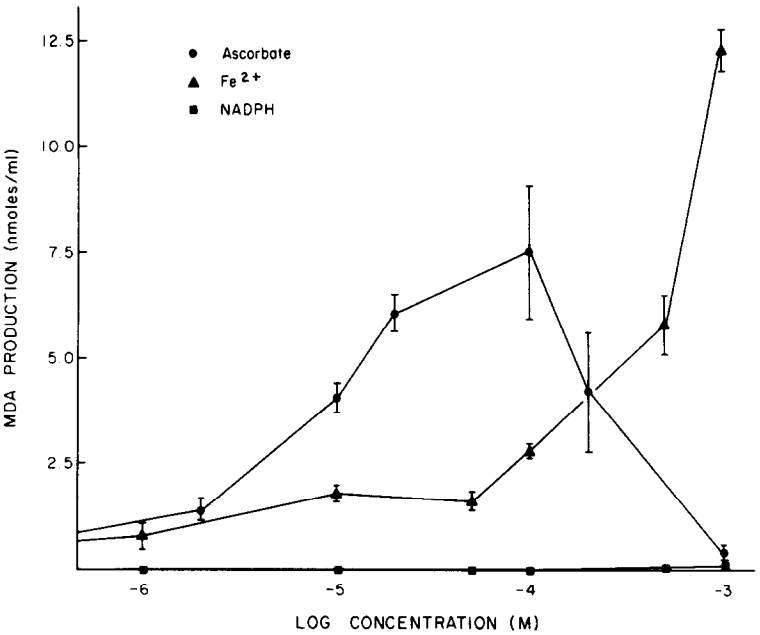

Fig. 1. Concentration-dependent stimulation of malonaldehyde (MDA) production by ascorbate, Fe^{2+} and NADPH in adrenal microsomes (0.25 mg protein/ml). Malonaldehyde was measured by the thiobarbituric acid test following a 60-min aerobic incubation at 37°C. Values are the means ± S.E. of 4—6 determinations.

shown in Fig. 2. After addition of Fe^{2+}, malonaldehyde production continued to increase for at least 90 min. In the presence of ascorbate, by contrast, malonaldehyde levels rapidly increased for approx. 30 min, but little additional change was seen during the next 60 min. We have established that malonaldehyde is not metabolized by adrenal microsomes under the incubation condi-

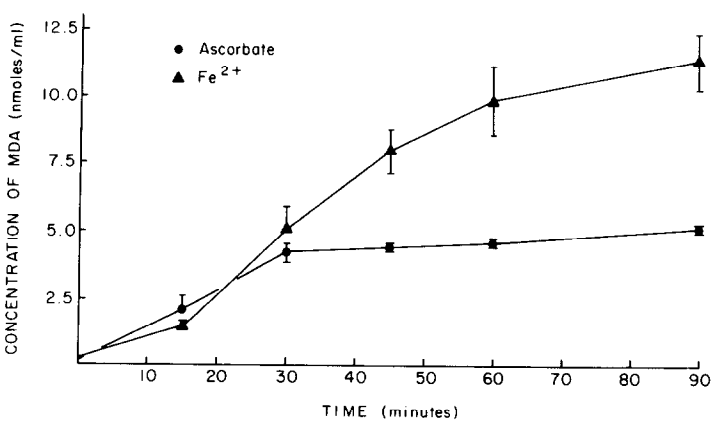

Fig. 2. Time-courses for Fe^{2+} (10^{-3} M) and ascorbate (10^{-4} M) stimulation of malonaldehyde (MDA) production by adrenal microsomes (0.25 mg protein/mg) incubated aerobically at 37°C for 60 min. Values are the means ±S.E. of 4—6 determinations.

tions described by adding known amounts prior to incubation and obtaining complete recovery after incubation. Therefore, the time courses reflect only malonaldehyde production.

Wills [15] has reported previously that in rat liver microsomes, Fe^{2+} and Fe^{3+} stimulate lipid peroxidation to about the same extent. We obtained similar results in guinea-pig liver microsomes using Fe^{2+} and Fe^{3+} concentrations ranging from 10^{-5} to 10^{-3} M (Table I). However, in guinea-pig adrenal microsomes, Fe^{3+} had relatively little effect on malonaldehyde production (Table I). The effects of Fe^{2+} on malonaldehyde production were far greater in adrenal than in hepatic microsomes. When expressed per mg of microsomal protein, adrenal production of malonaldehyde was approx. 5-times greater than hepatic production.

NADPH, when added alone to adrenal microsomes, did not affect malonaldehyde production (Fig. 1). However, in the presence of small amounts ($1 \cdot 10^{-6}$ M) of Fe^{2+}, NADPH produced a concentration-dependent stimulation of lipid peroxidation (Table II). The absence of this effect in heat-inactivated microsomes suggested that this stimulation was an enzymatic process. NADPH also enhanced, but to a lesser extent, the effects of Fe^{3+} on malonaldehyde production by adrenal microsomes (Table III), perhaps by promoting it's conversion to Fe^{2+}. In contrast to the stimulatory effect of NADPH on lipid peroxidation in the presence of low Fe^{2+} levels, NADPH diminished the effect of $1 \cdot 10^{-3}$ M Fe^{2+} on malonaldehyde production (Table IV). Inhibition of lipid peroxidation by NADPH in the presence of high Fe^{2+} concentrations was concentration-dependent and fully demonstrable in heat-treated microsomes, suggesting a non-enzymatic effect. The actions of ascorbate ($1 \cdot 10^{-4}$ M) on adrenal microsomal malonaldehyde production were similarly inhibited by NADPH in both normal and heat-treated microsomes (data not shown).

Interactions between ascorbate and iron were also observed. Ascorbate ($5 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$ M) synergistically interacted with low levels ($1 \cdot 10^{-6}$ M) of Fe^{2+} to enhance malonaldehyde production by adrenal microsomes (Table V). Malonaldehyde formation in the presence of both ascorbate and Fe^{2+} was

TABLE I

EFFECTS OF Fe^{2+} AND Fe^{3+} ON MALONALDEHYDE PRODUCTION BY LIVER AND ADRENAL MICROSOMES

Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Microsomes were incubated at optimal protein concentrations for lipid peroxidation (liver at approx. 0.75 mg/ml; adrenal at 0.25 mg/ml). Values are the means ±S.E. of 4–6 determinations and are expressed as nmol malonaldehyde/ml.

	Concentration of Fe^{2+} or Fe^{3+} (M)			
	$1 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	$1 \cdot 10^{-3}$
Liver				
Fe^{2+}	1.7 ± 0.2	1.6 ± 0.1	1.9 ± 0.1	6.8 ± 0.1
Fe^{3+}	1.6 ± 0.2	1.8 ± 0.1	2.3 ± 0.2	5.5 ± 0.2
Adrenal				
Fe^{2+}	1.8 ± 0.2	2.8 ± 0.2	5.8 ± 0.8	12.3 ± 0.4
Fe^{3+}	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.8 ± 0.2

TABLE II

CONCENTRATION-DEPENDENT STIMULATION OF MALONALDEHYDE PRODUCTION BY NADPH IN THE PRESENCE OF 10^{-6} M Fe^{2+} IN NORMAL OR HEAT-TREATED ADRENAL MICROSOMES

Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Values are the means ±S.E. of 4—6 determinations and are expressed as nmol malonaldehyde/ml.

	Concentration of NADPH (M)				
	$5 \cdot 10^{-5}$	$7.5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$2.5 \cdot 10^{-4}$	$5 \cdot 10^{-4}$
Normal microsomes	1.4 ± 0.2	2.8 ± 0.6	4.1 ± 0.5	6.9 ± 0.8	7.4 ± 0.6
Heat-treated microsomes	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.6 ± 0.3

TABLE III

CONCENTRATION-DEPENDENT STIMULATION OF MALONALDEHYDE PRODUCTION BY Fe^{3+} IN THE PRESENCE OR ABSENCE OF NADPH ($5 \cdot 10^{-4}$ M) IN ADRENAL MICROSOMES

Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Values are the means ±S.E. of 4—6 determinations and are expressed as nmol malonaldehyde/ml.

	Concentration of Fe^{3+} (M)					
	$1 \cdot 10^{-6}$	$5 \cdot 10^{-6}$	$1 \cdot 10^{-5}$	$5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-3}$
Fe^{3+} alone	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	1.1 ± 0.4
Fe^{3+} + NADPH	0.4 ± 0.1	0.9 ± 0.2	1.7 ± 0.4	2.7 ± 0.8	2.9 ± 0.7	2.0 ± 0.4

TABLE IV

EFFECTS OF NADPH ON Fe^{2+} (10^{-3} M) STIMULATION OF MALONALDEHYDE PRODUCTION IN NORMAL OR HEAT-TREATED ADRENAL MICROSOMES

Malonaldehyde was determined following a 60-min aerobic incubation at 37°C. Values are expressed as mean percentage of control ±S.E.; 4—6 determinations per value.

	Concentration of NADPH (M)					
	0	$5 \cdot 10^{-5}$	$7.5 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$2 \cdot 10^{-4}$	$5 \cdot 10^{-4}$
Normal microsomes	100	87 ± 4	78 ± 1	38 ± 9	21 ± 4	10 ± 1
Heat-treated microsomes	100	77 ± 7	67 ± 6	56 ± 4	6 ± 4	8 ± 1

TABLE V

EFFECTS OF ASCORBATE ON Fe^{2+} OR Fe^{3+} STIMULATION OF MALONALDEHYDE PRODUCTION BY ADRENAL MICROSOMES

Malonaldehyde production was determined following a 60-min aerobic incubation at 37°C. Values are the means ±S.E. of 4—6 determinations and are expressed as nmol malonaldehyde/ml.

	Concentration of Fe^{2+} or Fe^{3+}	
	$1 \cdot 10^{-6}$ M	$1 \cdot 10^{-3}$ M
Fe^{2+}		
Fe^{2+} alone	1.2 ± 0.4	13.9 ± 1.6
Ascorbate (10^{-4} M) alone	5.2 ± 2.5	5.2 ± 2.5
Fe^{2+} + ascorbate	15.7 ± 1.6	18.2 ± 3.2
Fe^{3+}		
Fe^{3+} alone	0.6 ± 0.2	1.2 ± 0.4
Ascorbate (10^{-4} M) alone	5.2 ± 2.5	5.2 ± 2.5
Fe^{3+} + ascorbate	12.5 ± 1.2	11.1 ± 2.5

greater than the summation of the individual effects of each. At high concentrations of Fe^{2+}, the synergism was not demonstrable. In contrast, at all concentrations of Fe^{3+} studied, ascorbate synergistically increased the production of malonaldehyde (Table V). Although Fe^{3+} alone had little effect on adrenal microsomal lipid peroxidation, in the presence of ascorbate large amounts of malonaldehyde were produced.

Discussion

The results indicate that a variety of substances affect lipid peroxidation in adrenal microsomes and that control of lipid peroxidation in adrenal and hepatic microsomes differ in several ways. Liver and adrenal are similar in that Fe^{2+} and ascorbate are potent inducers of non-enzymatic lipid peroxidation in both tissues. The capacity for non-enzymatic malonaldehyde production was far greater in adrenal than liver microsomes, perhaps as a result of the high concentration of unsaturated fatty acids in adrenal tissue [16]. In rats, the extent of lipid peroxidation is also greater in the adrenal than in the liver [17]. Malonaldehyde production was directly proportional to the Fe^{2+} concentration in adrenal microsomes but the response to ascorbate was maximal at a concentration of approx. 10^{-4} M. Higher concentrations of ascorbate produced a rapid decline in lipid peroxidation. Studies are now needed to determine the relationship between ascorbate oxidation by adrenal microsomes and the resulting rates of lipid peroxidation under various experimental conditions. It is possible that at high concentrations, the antioxidant properties of ascorbate may be manifested, thereby diminishing lipid peroxidation. The normal concentration of ascorbate in the guinea-pig adrenal is well above 10^{-4} M [28]. However, stimulation of the adrenal cortex by adrenocorticotropin diminishes adrenal ascorbate levels [29] and may thereby promote lipid peroxidation.

The effects of Fe^{3+} on lipid peroxidation differed considerably in guinea-pig adrenal and hepatic microsomes. In the liver, Fe^{3+} and Fe^{2+} were equipotent in stimulating malonaldehyde production. Similar results have been obtained with rat liver microsomes [15]. In the adrenal, however, Fe^{3+} had virtually no effect on lipid peroxidation. It has been proposed that Fe^{3+} does not directly affect lipid peroxidation but must first be reduced to Fe^{2+} [27]. If that proposal is correct, the capacity for the reduction of Fe^{3+} must exist in hepatic but not in adrenal microsomes. The reason(s) for the differences in the two tissues is presently not known and will require further investigation.

In rat liver microsomes, NADPH has been shown to be a potent stimulus to lipid peroxidation [14]. Similarly, we have found that NADPH initiates the production of malonaldehyde by guinea-pig hepatic microsomes in the absence of exogenous Fe^{2+}. However, in guinea-pig adrenal microsomes, NADPH alone had no effect on lipid peroxidation. This contrasts with the observation of Kitabchi [16] who used bovine adrenal microsomes and found that omission of NADPH from the incubation medium decreased malonaldehyde production. However, Kitabchi also included 17-hydroxyprogesterone, a substrate for the microsomal 21-hydroxylase, in his incubations and did not determine what effects changes in steroid metabolism might have on lipid peroxidation. In addition, Kitabchi used microsomal preparations which had been lyophilized and

stored frozen; freshly prepared microsomes were used in all of our experiments.

Although we found that NADPH alone had no effect on adrenal microsomal lipid peroxidation, in the presence of small amounts of Fe^{2+}, NADPH stimulated malonaldehyde production. The stimulatory effect is probably enzymatic since it was not demonstrable in heat-treated microsomes. It is possible that NADPH, acting through microsomal NADPH-dependent reductases, helps to maintain the iron in its reduced form (Fe^{2+}) during the course of incubation. The effect of NADPH to enhance Fe^{3+} stimulation of lipid peroxidation is consistent with this hypothesis. However, the effect of NADPH to increase malonaldehyde production in the presence of Fe^{3+} was far less than the effect of Fe^{2+} on lipid peroxidation, suggesting that mechanisms other than the reduction of Fe^{3+} may also be involved in the stimulation of lipid peroxidation by NADPH.

The most surprising finding in these studies was the inhibition of malonaldehyde production by NADPH in the presence of high Fe^{2+} concentrations. The inhibition is apparently non-enzymatic since the effect was fully demonstrable in heat-treated microsomes. Ascorbate-induced lipid peroxidation was similarly diminished by NADPH. In other studies [30], we have demonstrated that NADPH also inhibits the Fe^{2+}-stimulated oxidation of linoleic acid, suggesting that the NADPH molecule may act as a direct antioxidant. Thus, NADPH apparently exerts opposing effects on malonaldehyde production by adrenal microsomes and under any given set of conditions, the net effect is probably determined by the balance between these two opposing actions. The reason that the inhibitory effect of NADPH is manifested at high Fe^{2+} concentrations and the stimulatory effect at low Fe^{2+} concentrations is not clear. However, since that stimulatory effect of NADPH requires microsomal enzymatic activity, it is possible that high Fe^{2+} concentrations inhibit the enzyme(s) involved, shifting the balance in favor of the antioxidant properties of NADPH. We have found, for example, that high concentrations of Fe^{2+} produce substantial decreases in adrenal microsomal NADPH-cytochrome *c* reductase activity. Studies are now in progress to evaluate the rates of NADPH oxidation and to compare them with the rates of lipid peroxidation under the various experimental conditions described.

These observations indicate that multiple interactions between a variety of substances can have profound effects on lipid peroxidation in adrenal microsomes. Since all of the substances studied are normally found in adrenocortical cells, such interactions may be important in the physiological regulation of adrenal lipid peroxidation. The significance of lipid peroxidation as a factor in the control of normal adrenocortical function (steroidogenesis) has yet to be determined. However, increases in lipid peroxidation have been shown to promote the degradation of cytochromes *P*-450 and decrease the activities of cytochrome *P*-450-containing enzymes [16,18]. Since many of the enzymes required for adrenal steroidogenesis are cytochrome *P*-450-dependent [31], lipid peroxidation might affect steroid secretion by the adrenal cortex. Studies are now needed to further examine the regulation of lipid peroxidation in the adrenal cortex and to determine its relationship to hormone output. Such studies should contribute to a better understanding of the mechanisms involved in the regulation of adrenocortical function.

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