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LIPID PEROXIDATION IN GUINEA PIG LUNG MICROSOMES

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Summary

The effects of substances known to influence lipid peroxidation were studied in guinea pig lung microsomes by measuring the formation of malonaldehyde in vitro. Incubation of lung microsomes at 37°C results in lipid peroxidation which appears to be an enzymatic process but is not dependent upon iron. Lipid peroxidation can be initiated non-enzymatically in lung microsomes by Fe^{2+}, but ascorbate and Fe^{3+} have very little effect on malonaldehyde formation. The effects of NADPH on lipid peroxidation are dependent upon the concentration of Fe^{2+} in the incubation medium. At concentrations of Fe^{2+} between 0.05 mM and 1 mM, addition of NADPH causes an increase in lipid peroxidation over that produced by Fe^{2+} alone. This stimulation by NADPH is an enzymatic process and phosphate is required for the maximal effect. Addition of NADPH to lung microsomes in the presence of Fe^{3+} does not increase malonaldehyde formation over that produced by Fe^{3+} alone, suggesting that NADPH does not influence lipid peroxidation by maintaining iron in the reduced form. At concentrations of Fe^{2+} greater than 1 mM, NADPH inhibits Fe^{2+}-induced lipid peroxidation in normal microsomes and in microsomes in which enzymes have been inactivated with heat. This latter result suggests that the inhibition by NADPH is at least partially non-enzymatic. The results of all of these experiments are discussed and compared with those obtained during lipid peroxidation in liver microsomes. We conclude that the processes involved in pulmonary microsomal lipid peroxidation differ significantly from those in hepatic microsomes.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfomic acid.

Introduction

Lipid peroxidation has been shown to occur in vivo in the lung following exposure to oxidant gases or to various other substances that reach the lung via the blood stream. For example, exposure to ozone [1—3] and to nitrogen dioxide [4] leads to an increase in the level of lipid peroxidation in the lung. Furthermore, it has been suggested that the level of ozone found in the air in major urban areas is sufficient to initiate this process in vivo [5]. Even high partial pressures of oxygen can cause formation of free radicals which, in turn, may lead to lipid peroxidation [6]. Peroxidation of lung lipids can also be produced by substances other than oxidant gases. Paraquat is an herbicide which, when ingested or injected parenterally, causes extensive lung damage [7]. Bus et al. [8] have provided evidence to indicate that paraquat causes lipid peroxidation of lung tissue in vivo. Thus, these studies show that the process of lipid peroxidation is associated with certain pathological conditions and biochemical changes which occur in the lung.

Although the lipid peroxidation which occurs in vivo in the lung following exposure to oxidant gases has been investigated, very little is known about factors affecting in vitro lipid peroxidation in pulmonary microsomes [1—8]. However, in vitro lipid peroxidation has been studied extensively in hepatic microsomes and can be initiated in a variety of ways. For example, peroxidation of microsomal lipids can be induced by NADPH in the presence of ferrous or ferric iron [9—11]. NADPH-induced lipid peroxidation has been found to be closely associated with the microsomal enzyme system which catalyzes oxidative drug metabolism in that both lipid peroxidation and the drug-metabolizing system utilize the enzyme, NADPH-cytochrome *c* reductase [12,13]. Thus, NADPH-induced lipid peroxidation is referred to as enzymatic lipid peroxidation. Formation of lipid peroxides in hepatic microsomes can also be initiated by ascorbate [9,11] or by ferrous or ferric iron [14]. Both ascorbate- and iron-induced peroxidation of microsomal lipids are unaffected by enzyme inactivation and are, therefore, referred to as non-enzymatic lipid peroxidation.

The objective of this investigation was to study the effects of substances known to influence in vitro lipid peroxidation, i.e., NADPH, Fe^{2+}, Fe^{3+}, and ascorbate, on peroxidation of lipids in guinea pig lung microsomes. The results of the experiments presented here indicate that the processes involved in in vitro pulmonary microsomal lipid peroxidation differ in many ways from those in hepatic microsomes. A preliminary report of these results has appeared previously [15].

Materials and Methods

Male English short-hair guinea pigs (approx. 1 kg) were obtained from Camm Research Institute, Wayne, NJ. The animals used in this study were older guinea pigs (i.e., 'retired breeders'). We found that if younger animals which weighed less than 1 kg were used, the magnitude of the lipid peroxidation was greatly diminished. The animals were killed by decapitation and the lungs were dissected free of the trachea, bronchi, and connective tissue. The lungs were finely minced by chopping four times with a McElwain Tissue Chopper (The

Mickle Engineering Co., Gomshall, Surrey, U.K.) which was set for a slice thickness of 5 mm. The mince was then homogenized in a solution containing 0.154 M KCl and 0.05 M Tris-HCl (pH 7.4) using a Teflon-glass Potter-Elvjehem homogenizer and microsomes were obtained by differential centrifugation [16]. In some experiments microsomes were prepared with 3 mM ethylenediaminetetraacetate (EDTA) included in the homogenization medium (EDTA-prepared prepared microsomes) in order to eliminate endogenous iron. In some experiments microsomal enzymes were inactivated by heating the microsomal suspension (250 mg lung tissue/ml) at 70°C for 2 min. These microsomes are referred to as heat-treated microsomes. No metabolism of benzphetamine, which was measured as described previously by Hook et al. [17], could be demonstrated in heat-treated microsomes. For all experiments the microsomes, either normal or heat treated, were resuspended in 0.1 M phosphate buffer (0.081 M K_2HPO_4 and 0.019 M KH_2PO_4; pH 7.4) at a final concentration equivalent to 100 mg lung tissue/ml. The protein content of the microsomal suspensions was 1.7 (±0.2) mg of microsomal protein/ml (mean value for nine determinations).

Oxygen (100%) was bubbled through the suspension of microsomes for 1 min prior to the start of the experiment. The incubation medium consisted of 2.5 ml of the microsomal suspension and the appropriate concentration(s) of the substance(s) used to initiate or modify lipid peroxidation. The substances used to initiate lipid peroxidation were NADPH (type I), L-ascorbic acid (Sigma Chemical Co., St. Louis, MO), $FeSO_4$, and $FeCl_3$. The samples were incubated at 37°C for various lengths of time. The lipid peroxidation which occurred during the incubation period was measured as the amount of malonaldehyde formed by the lung microsomes. Malonaldehyde was measured according to the method of Ottolenghi [18] as modified by Hunter et al. [19]. After the incubation period, the samples were placed on ice. After cooling, 0.3 ml of 5 N HCl and 0.625 ml of 40% trichloroacetic acid were added to each sample. Thiobarbituric acid (0.625 ml of a 2% solution) was added and the samples were incubated at 90°C for 20 min. After this incubation, the samples were placed on ice for 5 min and then centrifuged at 30 000 × *g* in a Sorvall Model SS-3 centrifuge (Ivan Sorvall Co., Norwalk, CN) for 5 min. 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 (Gilford Instrument Co., Oberlin, OH) and by using a molar extinction coefficient, ϵ, of $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ [20].

Results

Lipid peroxidation during incubation of microsomes

There is a small amount of malonaldehyde present in freshly prepared, unincubated lung microsomal suspensions. Incubation of the microsomes at 37°C results in formation of additional malonaldehyde which becomes maximal after 60–90 min. The magnitude of the lipid peroxidation which occurs during incubation and the effect of inactivation of microsomal enzymes (by heat treatment) on this process are shown in Table I. Incubation of the suspensions results in lipid peroxidation only in normal microsomes and not in the heat-

TABLE I

LIPID PEROXIDATION IN UNINCUBATED MICROSOMES AND IN LUNG MICROSOMES INCUBATED AT 37°C

For incubated samples malonaldehyde levels were measured after incubation of the microsomal suspensions at 37°C for 2 h. Unincubated samples were stored at 4°C during the 2 h incubation period. EDTA was added to the microsomal suspensions prior to the incubation period. In the experiments with Hepes buffer, the microsomes were suspended in Hepes rather than in phosphate buffer. In all other experiments the microsomes were suspended in phosphate buffer. The numbers shown are mean values for six experiments ± S.E.

Treatment (concn.)	Lipid peroxidation (nmol malonaldehyde/mg microsomal protein)	
	Unincubated microsomes	Incubated microsomes
Control	0.34 ± 0.02	0.70 ± 0.02
Heat treatment	0.39 ± 0.01	0.41 ± 0.01
EDTA (1 mM)	0.28 ± 0.02	0.69 ± 0.03
Hepes buffer	0.39 ± 0.08	0.66 ± 0.03

treated preparations. In liver microsomes iron is required for lipid peroxidation and malonaldehyde formation is much greater in phosphate buffer than in buffers without phosphate [9—11]. Therefore, we tested the effects of chelation of iron (with EDTA) and suspension of the microsomes in Hepes buffer on lipid peroxidation in lung microsomes. The results are also shown in Table I. Neither EDTA nor incubation in Hepes rather than in phosphate buffer has any effect on the formation of malonaldehyde. These results indicate that the lipid peroxidation which occurs during incubation alone is an enzymatic process and does not require iron or phosphate.

Non-enzymatic lipid peroxidation

Non-enzymatic lipid peroxidation can be initiated in hepatic microsomes by ferrous or ferric iron or by ascorbate. The effects of these substances on lung microsomal lipid peroxidation are shown in Table II. Addition of 2.5 mM Fe^{3+}

TABLE II

NON-ENZYMATIC LIPID PEROXIDATION IN LUNG MICROSOMES

Values for malonaldehyde formation were obtained following incubation of the microsomal suspensions at 37°C for 2 h. The control value is that lipid peroxidation which occurs as a result of incubation alone. All experiments except for one were done with microsomes suspended in phosphate buffer. The exception is the experiment in which microsomes were suspended in Hepes buffer (last row). The numbers shown are mean values for six experiments ± S.E.

Treatment (concn.)	Lipid peroxidation (nmol malonaldehyde/mg microsomal protein)
Control	0.77 ± 0.06
Fe^{2+} (2.5 mM)	7.50 ± 0.19
Ascorbate (0.25 mM)	1.17 ± 0.14
Fe^{3+} (2.5 mM)	0.97 ± 0.05
Ascorbate + Fe^{2+}	3.56 ± 0.90
Ascorbate + Fe^{3+}	1.64 ± 0.25
Fe^{2+} (in Hepes buffer)	1.43 ± 0.20

or 0.25 mM ascorbate, concentrations which produce maximal effects, to lung microsomes causes very little lipid peroxidation. However, addition of 2.5 mM Fe^{2+}, an amount which produces the maximal effect, yields a 10-fold increase in malonaldehyde formation over that which occurs during incubation alone. The Fe^{2+}-induced lipid peroxidation becomes maximal after approx. 75 min of incubation. As shown in Table II, Fe^{2+}-induced lipid peroxidation is much greater in phosphate than in Hepes buffer. However, this is not surprising since other investigators have suggested that Fe^{2+}-phosphate complexes are very effective in promoting lipid peroxidation [11,14,21].

In hepatic microsomes and mitochondria the lipid peroxidation induced by either Fe^{2+} or Fe^{3+} can be enhanced by the addition of ascorbate [11,18]. The effects of adding Fe^{3+} and ascorbate or Fe^{2+} and ascorbate on lung microsomal lipid peroxidation are also shown in Table II. The magnitude of the lipid peroxidation which occurs in the presence of both ascorbate and Fe^{3+} is approximately equal to the sum of their individual effects. On the other hand, ascorbate causes inhibition of Fe^{2+}-induced lipid peroxidation. This inhibition may be due to the antioxidant properties of the ascorbate molecule.

Effects of NADPH on lipid peroxidation

Since hepatic microsomal lipid peroxidation can be stimulated by NADPH, its effect on malonaldehyde formation in guinea pig lung microsomes was studied. Lung microsomal lipid peroxidation is stimulated by NADPH and the magnitude of this effect is shown in Table III. The concentration of NADPH used in these experiments (0.4 mM) is one which produces a maximal effect. Malonaldehyde formation reaches a maximal level after approx. 90 min of incubation. The effect of inactivation of microsomal enzymes on the ability of NADPH to stimulate lipid peroxidation was also studied (Table III). NADPH stimulates lipid peroxidation in normal microsomes but not in heat-treated preparations. These results indicate that NADPH-stimulated lipid peroxidation in lung microsomes is an enzymatic process.

TABLE III

NADPH-INDUCED LIPID PEROXIDATION IN LUNG MICROSOMES

Malonaldehyde levels were measured after incubation of the microsomal suspensions at 37°C for 2 h. The control values represent the lipid peroxidation which occurs as a result of incubation alone. The microsomes were suspended in either phosphate or Hepes buffer. EDTA was added to the microsomal suspensions just prior to the incubation period. The numbers are mean values for six experiments ± S.E.

Treatment (concn.)	Lipid peroxidation (nmol malonaldehyde/mg microsomal protein)	
	Phosphate buffer	Hepes buffer
Control	0.70 ± 0.02	0.66 ± 0.03
NADPH (0.4 mM)	2.41 ± 0.30	0.63 ± 0.03
Heat-treated microsomes + NADPH	0.48 ± 0.01	—
NADPH + EDTA (1 mM)	0.53 ± 0.03	—
Fe^{2+} (0.1 mM)	—	1.00 ± 0.05
Fe^{2+} (0.1 mM) + NADPH	—	0.96 ± 0.05

NADPH-stimulated lipid peroxidation in liver microsomes occurs only in the presence of iron [9—11]. It is possible that iron is required for NADPH-stimulated lipid peroxidation in lung microsomes and that a sufficient amount is present as a contaminant in the phosphate buffer. Therefore, we studied the effects of phosphate buffer, EDTA, and addition of Fe^{2+} on NADPH-stimulated lipid peroxidation in lung microsomes (Table III). NADPH-stimulated lipid peroxidation occurs in phosphate buffer but not in microsomes suspended in Hepes buffer. In addition, NADPH does not stimulate malonaldehyde formation in the presence of EDTA. These results suggest that Fe^{2+} is necessary for NADPH-induced lipid peroxidation and a sufficient amount is present in the phosphate buffer. However, in other experiments (Table III) we found that addition of 0.1 mM Fe^{2+}, an amount much greater than that found in phosphate buffer, to pulmonary microsomes in Hepes buffer does not bring NADPH-stimulated lipid peroxidation up to the level obtained in phosphate buffer. Thus, both Fe^{2+} and phosphate are required for maximal activity and it is probably the Fe^{2+}-phosphate complexes which are responsible for the NADPH-dependent lipid peroxidation in phosphate buffer.

Since Fe^{2+} appears to be required for NADPH-induced lipid peroxidation, the effects of 0.4 mM NADPH at various Fe^{2+} concentrations were studied. The

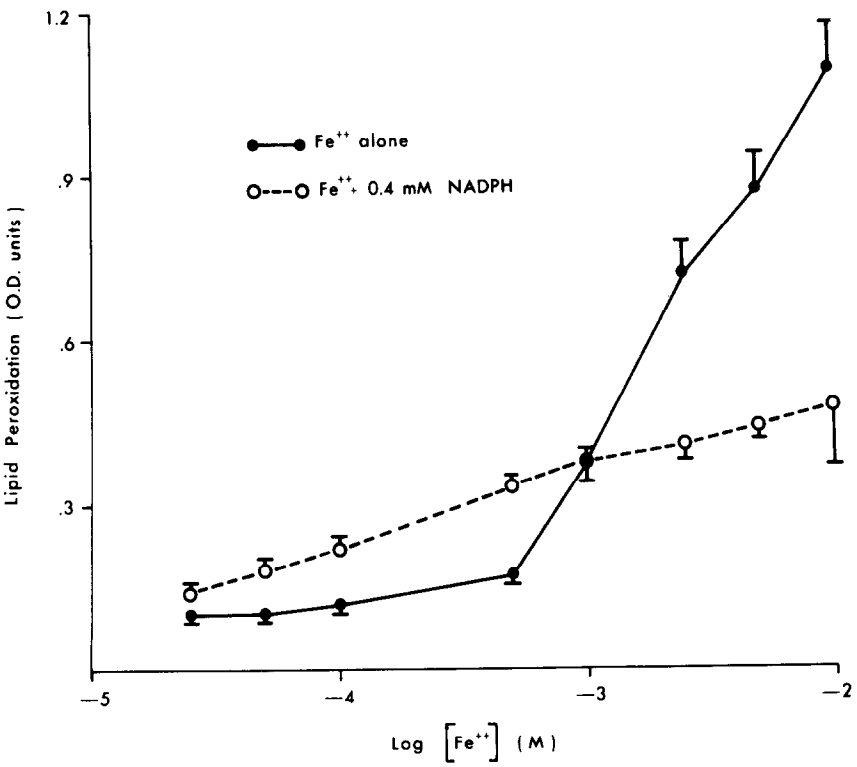

Fig. 1. Effect of NADPH on lipid peroxidation at various iron concentrations. EDTA-prepared microsomes were used for these experiments. The values were obtained after an incubation period of 2 h at 37° C. The points represent mean values for six experiments and the bars indicate the S.E.

results are shown in Fig. 1. In these experiments EDTA-prepared microsomes were used to eliminate the endogenous iron. NADPH can either enhance or inhibit the lipid peroxidation induced by ferrous iron, depending upon the Fe^{2+} concentration. At Fe^{2+} concentrations between 0.05 mM and 1 mM, lipid peroxidation in the presence of NADPH and Fe^{2+} is greater than that produced by Fe^{2+} alone; i.e., NADPH-stimulated lipid peroxidation is observed at the lower Fe^{2+} concentrations. However, at Fe^{2+} levels greater than 1 mM, NADPH inhibits Fe^{2+}-induced lipid peroxidation. The remainder of the experiments presented here were designed to further study these different effects of NADPH at various Fe^{2+} concentrations.

It has been proposed by other investigators that stimulation of lipid peroxidation by NADPH in the presence of low Fe^{2+} concentrations occurs because NADPH maintains iron in the reduced form, i.e., Fe^{2+} [22—24]. This hypothesis is supported by the finding in rat liver microsomes that Fe^{3+} alone does not stimulate lipid peroxidation, but Fe^{3+} in the presence of NADPH is an effective initiator of lipid peroxidation, presumably because Fe^{3+} is converted to Fe^{2+} [23]. In order to determine if the function of NADPH in guinea pig lung microsomes is to maintain iron in the reduced form, similar types of experiments were conducted. It has already been shown that Fe^{3+} alone, in concentrations as high as 2.5 mM, produces very little lipid peroxidation (Table II). The effects of NADPH at various concentrations of Fe^{3+} were also studied (Table IV). The lipid peroxidation initiated by NADPH in combination with Fe^{3+} is not significantly different from that induced by Fe^{3+} alone. Therefore, lung microsomes are unlike liver microsomes in that NADPH in the presence of Fe^{3+} is not an effective initiator of lipid peroxidation. It seems possible then that the function of NADPH in guinea pig lung microsomes is something other than the maintenance of iron in the reduced form.

As shown in Fig. 2, NADPH inhibits Fe^{2+}-induced lipid peroxidation when the iron concentration is greater than 1 mM. Since inhibition of lipid peroxidation by NADPH has not been previously reported, we decided to further study this phenomenon. A dose-response curve for the effect of NADPH on 2.5 mM Fe^{2+}-induced lipid peroxidation is shown in Fig. 2. The lowest concentration of NADPH to inhibit Fe^{2+}-induced lipid peroxidation is approx. 0.001 mM and

TABLE IV

EFFECT OF Fe^{3+} AND Fe^{3+} PLUS NADPH ON LIPID PEROXIDATION IN LUNG MICROSOMES

Malonaldehyde levels were measured after incubation of the microsomal suspensions at 37°C for 2 h. EDTA-prepared microsomes were used for these experiments. All experiments were done with the microsomes suspended in phosphate buffer. The numbers shown are mean values for seven experiments ± S.E.

Concn. of Fe^{3+} (mM)	Lipid peroxidation (nmol malonaldehyde/mg microsomal protein)	
	Fe^{3+} alone	Fe^{3+} + 0.4 mM NADPH
0.001	0.55 ± 0.10	0.54 ± 0.04
0.1	0.70 ± 0.07	0.83 ± 0.11
1	0.89 ± 0.09	1.02 ± 0.11
2.5	0.87 ± 0.08	1.03 ± 0.10

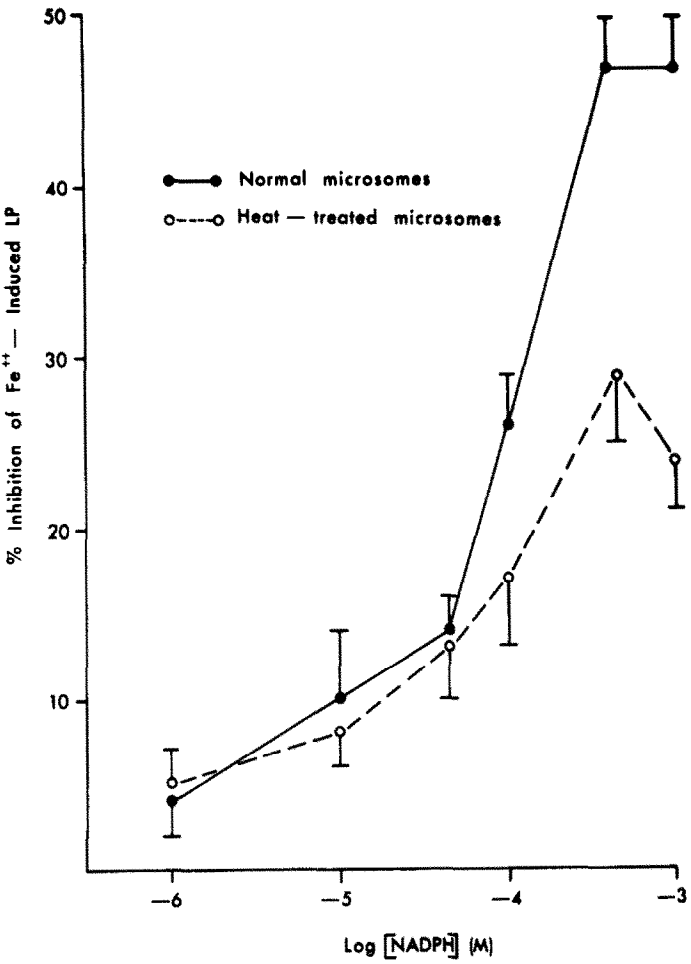

Fig. 2. Dose-response relationship for NADPH inhibition of 2.5 mM Fe^{2+}-induced lipid peroxidation (LP) in normal and heat-treated microsomes. The values were obtained after an incubation period of 2 h at 37° C. The points represent mean values for six experiments and the bars indicate the S.E.

the maximal inhibition of 45—50% occurs at about 0.4 mM NADPH. In order to determine whether or not this inhibition requires enzymatic activity, the effects of NADPH on Fe^{2+}-induced lipid peroxidation were studied in heat-treated microsomes (Fig. 2). NADPH inhibition of malonaldehyde formation also occurs in heat-treated microsomes, indicating that at least part of the inhibition produced by NADPH does not require enzymatic activity.

Discussion

These experiments represent the first in-depth investigation of the factors affecting pulmonary microsomal lipid peroxidation. The results demonstrate that there are many differences between pulmonary and hepatic microsomal lipid peroxidation and also provide additional information about some of the processes which had been observed previously in liver microsomes. For exam-

ple, incubation of pulmonary microsomes at 37°C results in formation of malonaldehyde. Lipid peroxidation during incubation of liver microsomes has also been observed [11], but this process has not been studied extensively. In lung, this process may be enzymatic since heat inactivation of microsomal enzymes abolishes it. The identity of the enzymes involved is not known. However, NADPH-linked reactions are probably not involved since EDTA inhibits NADPH-induced lipid peroxidation but has no effect on the malonaldehyde formation which occurs during incubation of the microsomes. This latter observation also indicates that the lipid peroxidation which occurs during microsomal incubation alone is not dependent upon iron. This is most unusual, since almost all lipid peroxidation which has been observed in biological tissues seems to require iron.

The effects of ascorbate and Fe^{3+} on lipid peroxidation are quite different in pulmonary and hepatic microsomes. Wills [11,14] has reported that ascorbate and Fe^{3+} stimulate formation of malonaldehyde in liver microsomes. However, these substances have very little effect on pulmonary microsomal lipid peroxidation. In addition, ascorbate enhances the lipid peroxidation induced by either Fe^{2+} or Fe^{3+} in hepatic microsomes [11,18]. In lung microsomes ascorbate actually inhibits the malonaldehyde formation induced by Fe^{2+}. The reasons for these differences are presently unknown and will require further investigation.

NADPH stimulates lung microsomal lipid peroxidation when Fe^{2+} is present in concentrations less than 1 mM. This stimulation is similar to NADPH-induced lipid peroxidation in liver microsomes in that it is enzymatic and dependent upon the presence of iron and phosphate in the incubation medium. However, the amount of NADPH-dependent lipid peroxidation which occurs in lung microsomes is much less than that produced in liver microsomes. We have measured NADPH-induced malonaldehyde formation in rat liver microsomes and found the levels to be 5—10-fold greater than in guinea pig lung [25]. Recently, Willis and Recknagel [26] have found that NADPH stimulates lipid peroxidation in rat lung microsomes and that this stimulation is less than that seen in liver microsomes. The reason for lower levels of NADPH-dependent lipid peroxidation in lung microsomes is unknown, but it may be due in part to the differences in enzyme activities, since NADPH-cytochrome *c* reductase activity is lower in lung than in liver microsomes [27].

There are several effects of NADPH on lung microsomal lipid peroxidation which are quite different from those reported in liver microsomes. For example, in lung microsomes NADPH can either stimulate or inhibit Fe^{2+}-induced lipid peroxidation, depending on the concentration of iron present. NADPH stimulates lipid peroxidation only when iron is present in concentrations less than 1 mM. It has been proposed that NADPH stimulates lipid peroxidation in liver microsomes by maintaining iron in the reduced state, i.e., in the Fe^{2+} form [22,23]. However, in lung microsomes neither Fe^{3+} alone nor Fe^{3+} in the presence of NADPH are effective initiators of lipid peroxidation. Therefore, it appears that NADPH does not function to maintain iron in the reduced form in lung microsomes and must stimulate lipid peroxidation by some other mechanism.

NADPH inhibits Fe^{2+}-induced lipid peroxidation in pulmonary microsomes

when iron is present in concentrations greater than 1 mM. This is, to the best of our knowledge, the first demonstration of NADPH inhibition of microsomal lipid peroxidation. The NADPH inhibition still occurs in heat-treated microsomes, indicating that the inhibition is at least partially non-enzymatic. We have also found that NADPH inhibits the Fe^{2+}-stimulated oxidation of linoleic acid [15]. Therefore, the non-enzymatic inhibition may be due to direct antioxidant properties of the NADPH molecule or to some property of NADPH which may allow it to bind to iron and alter the concentration of free iron available to induce lipid peroxidation.

In summary, the results of all of these experiments indicate that the processes involved in pulmonary microsomal lipid peroxidation differ significantly from those in hepatic microsomes. In particular, the effects of NADPH in pulmonary and liver microsomes are quite different. We have demonstrated for the first time that NADPH can inhibit ferrous iron-stimulated lipid peroxidation in lung microsomes. Furthermore, the characteristics of pulmonary lipid peroxidation described in this communication are not perculiar to guinea pig lung microsomes. We have made similar observations in lung microsomes obtained from rats with one exception; incubation of rat lung microsomes does not result in formation of malonaldehyde. Therefore, the observed differences between lung and liver microsomal lipid peroxidation do not reflect a species dependence.

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