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The stimulatory effects of asbestos on NADPH-dependent lipid peroxidation in rat liver microsomes

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Lipid peroxidation in rat liver microsomes induced by asbestos fibres, crocidolite and chrysotile, is greatly increased in the presence of NADPH, leading to malondialdehyde levels comparable with those induced by CCl_4, a very strong inducer of lipid peroxidation. This synergic effect only occurs during the first minutes and could be explained by an increase or a regeneration of the ferrous active sites of asbestos by NADPH, which in turn could rapidly be prevented by the adsorption of microsomal proteins on the surface of the fibres. It is not inhibited by superoxide dismutase, catalase and mannitol, indicating that oxygen radicals are not involved in the reaction. It is also not inhibited by desferrioxamine, indicating that it is not due to a release of free iron ions in solution from the fibres. Lipid peroxidation in NADPH-supplemented microsomes is also greatly increased upon addition of magnetite. This could be linked to the presence of ferrous ions in this solid iron oxide, since the ferric oxides haematite and goethite are completely inactive.

INTRODUCTION

The asbestos minerals are fibrous silicates well known for their adverse effects on the respiratory system. After prolonged periods of inhalation, they produce fibrosis of the lung and two types of cancer, mesothelioma and bronchogenic carcinoma (Mossman *et al.*, 1983). They induce cell damage probably by modifying cellular membranes and their haemolytic action has been known for several years (Mossman *et al.*, 1983). These effects could be due to their ability to increase lipid peroxidation inside the cells. Accordingly, it was recently reported that asbestos fibres induced moderate lipid peroxidation on aerobic incubation with either soybean lipids (Weitzman & Weitberg, 1985) or liver microsomes (Gulumian *et al.*, 1983). The low levels of lipid peroxidation detected in these studies could be due to the absence of reducing equivalents in the lipid or microsomal suspensions. Actually, asbestos fibres were reported to have sites on their surface which can transfer electrons to electron-poor substrates such as tetracyanoethylene (Bonneau & Pezerat, 1983). It was thus tempting to test their aptitude to act as catalysts in an enzymic chain of electron transfer leading to dioxygen reduction and to significant lipid peroxidation. To this end, the microsomal electron transport system seemed to be suitable as it was known, from previous studies using liver microsomes (Kandaswami *et al.*, 1986) as well as epithelial cells (Mossman *et al.*, 1983) that asbestos particles could interact with membrane-bound oxidases such as aryl hydrocarbon hydroxylase.

In the present paper, we report that lipid peroxidation in rat liver microsomes induced by crocidolite, the main amphibole of economic and health significance, or chrysotile, the serpentine asbestos, as well as magnetite, an iron oxide present as an impurity on chrysotile surface, is greatly increased in the presence of NADPH. Experiments are described in which the different possible roles of asbestos in this stimulation of lipid peroxidation are studied.

MATERIALS AND METHODS

Reagents

Two types of asbestos were used, crocidolite and Rhodesian A chrysotile, supplied by the Union Internationale Contre le Cancer. Magnetite, haematite and goethite were supplied by H.P. Superoxide dismutase and catalase were obtained from Sigma. Desferrioxamine B (mesylate salt) was purchased from CIBA-Geigy. Chemical compounds were obtained from regular commercial sources. Liver microsomes were prepared (Kremers *et al.*, 1981) from male Sprague–Dawley rats which had been pretreated with sodium phenobarbital (three daily doses of 80 mg/kg body wt. intraperitoneally). Protein concentration was determined as previously described (Lowry *et al.*, 1951).

Incubations

Microsomes were suspended in 0.1 M-phosphate buffer, pH 7.4, in the presence of 0.1 mM-EDTA, 12 mM-glucose 6-phosphate and 1 mM-$NADP^+$. The total volume was 1 ml and contained 0.6 or 1 mg of protein. The solution was incubated at 37 °C and the reaction started by adding 2 units of glucose-6-phosphate dehydrogenase for generating NADPH. Lipid peroxidation induced by asbestos fibres or CCl_4 was studied by previously adding 1, 5 or 10 mg of crocidolite or chrysotile or 2 μl of CCl_4 respectively. The reaction was stopped by adding 150 μl of ethanol containing 0.2% butylated hydroxytoluene after 0, 5, 15 and 30 min incubation. For asbestos pretreatment, microsomes

Abbreviation used: MDA, malondialdehyde.

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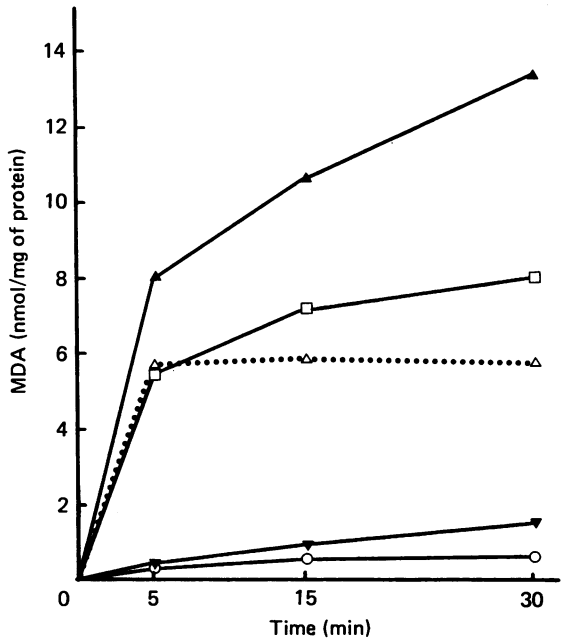


Fig. 1. Crocidolite-induced formation of MDA in rat liver microsomes

All incubations contained microsomes (0.6 mg of protein/ml), 12 mM-glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase and 0.1 mM-EDTA in 0.1 M-phosphate buffer, pH 7.4. Additional reagents were: ○, 10 mg of crocidolite fibres; ▼, 1 mM-$NADP^+$; □, 1 mM-$NADP^+$ and 10 mg of crocidolite; △, 1 mM-$NADP^+$, 10 mg of crocidolite and 1 mM-desferrioxamine; ▲, 1 mM-$NADP^+$ and 2 μl of CCl_4. MDA was measured by the thiobarbituric assay (Buege & Aust, 1978).

(0.6 mg/ml) were preincubated with crocidolite fibres (10 mg/ml) and the whole NADPH-generating system except glucose-6-phosphate dehydrogenase for 30 min at 37 °C. The reaction was then initiated by adding the enzyme and stopped immediately or 5 min later as described above.

Measurement of lipid peroxidation

Lipid peroxidation was measured by malondialdehyde (MDA) formation using the thiobarbituric assay (Buege & Aust, 1978). A freshly prepared solution (2 ml) containing trichloroacetic acid (15%), thiobarbituric acid (0.375%) and 0.25 M-HCl was added to the microsomal suspension. After stirring, the tubes were heated for 15 min in a boiling-water bath, cooled and centrifuged at 1800 *g* for 15 min. The amount of the coloured product was measured by determining the absorbance at 535 nm against a blank corresponding to the incubation of microsomes (0.6 mg of protein/ml) alone. An ϵ of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ was used.

NADPH oxidation

NADPH oxidation was measured in rat liver microsomes (0.1 mg of protein/ml) suspended in 0.1 M-phosphate buffer (pH 7.4)/0.1 mM-EDTA in 1 cm glass cuvettes. After addition of NADPH (final concentration 0.1 mM), the absorption change at 340 nm was recorded with an Aminco DW2 spectrophotometer at 30 °C. To study the effects of crocidolite on NADPH oxidation the following method was used: the same microsomal suspension was slowly introduced in the glass cuvette containing the fibres (10 mg/ml final concentration) and after 1 min NADPH (0.1 mM) was added and the absorption change of the solution was recorded at 340 nm. From the time curves obtained from four experiments the absorbance at 340 nm was measured at 0, 1, 2, 3, 4 and 5 min. An ϵ of 6220 $M^{-1} \cdot cm^{-1}$ was used.

RESULTS

Synergic effect of asbestos and NADPH on microsomal lipid peroxidation

Lipid peroxidation from rats pretreated with phenobarbital was measured by the formation of MDA. The results are shown in Fig. 1 and Table 1. As expected, no significant MDA was formed after 30 min upon aerobic incubation of rat liver microsomes in the absence of both NADPH and asbestos fibres. Addition of NADPH to the microsomal system, as expected, led to a slow but significant formation of MDA (Hochstein & Ernster, 1963; Aust & Svingen, 1982; Aust *et al.*, 1985). In the absence of NADPH, crocidolite fibres (10 mg/ml) only led to low amounts of MDA, in agreement with previous reports (Gulumian *et al.*, 1983). Lipid peroxidation was greatly and rapidly enhanced by adding both crocidolite and NADPH to the microsomal solution. The synergic effect seems to be very efficient only during the first 5 min, since the rate of lipid peroxidation rapidly decreased between 5 and 30 min. A second addition of 10 mg of fibres after 30 min incubation led to another burst of peroxidation (results not shown). It is interesting to note that high concentrations of CCl_4 (20 mM), one of the strongest known inducers of lipid

Table 1. Effect of crocidolite and chrysotile on lipid peroxidation in rat liver microsomes

Control reaction mixtures contained microsomes (0.6 mg of protein/ml), 12 mM-glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase and 0.1 mM-EDTA in 0.1 M-phosphate buffer, pH 7.4. Additional reagents were 1 mM-$NADP^+$, fresh fibres (crocidolite or chrysotile) and fibres preincubated with microsomes for 30 min, where indicated. MDA was measured as described in the Materials and methods section after 5 min incubation. Each value is the mean ± S.D. for four to ten observations.

	MDA produced (nmol/mg of protein)
Control	0
+NADPH	0.42±0.15
+Crocidolite (10 mg)	0.34±0.10
+NADPH+crocidolite (10 mg)	5.49±0.45
+NADPH+crocidolite (5 mg)	2.68±0.32
+NADPH+crocidolite (1 mg)	1.20±0.22
+NADPH+chrysotile (10 mg)	2.02±0.21
+NADPH+preincubated crocidolite (10 mg)	0.85±0.25

Table 2. Effect of superoxide dismutase, catalase and desferrioxamine on lipid peroxidation catalysed by crocidolite in NADPH-supplemented liver microsomes

Control reaction mixtures contained microsomes (0.6 mg of protein/ml), 12 mM-glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 0.1 mM-EDTA and 1 mM-$NADP^+$ in 0.1 M-phosphate buffer, pH 7.4. Additional reagents were crocidolite (10 mg), desferrioxamine (1 mM), superoxide dismutase (100 units/ml), catalase (150 units/ml) and mannitol (0.1 M) where indicated. MDA was measured as described in the Materials and methods section after 5 min incubation. Each value is the mean ± S.D. for four to ten observations.

	MDA produced (nmol/mg of protein)
Control	0.42 ± 0.15
+ Desferrioxamine	0.05 ± 0.02
+ Crocidolite	5.49 ± 0.45
+ Crocidolite + superoxide dismutase	5.56 ± 0.80
+ Crocidolite + catalase	5.50 ± 0.50
+ Crocidolite + desferrioxamine	5.60 ± 0.35
+ Crocidolite + mannitol	5.45 ± 0.45

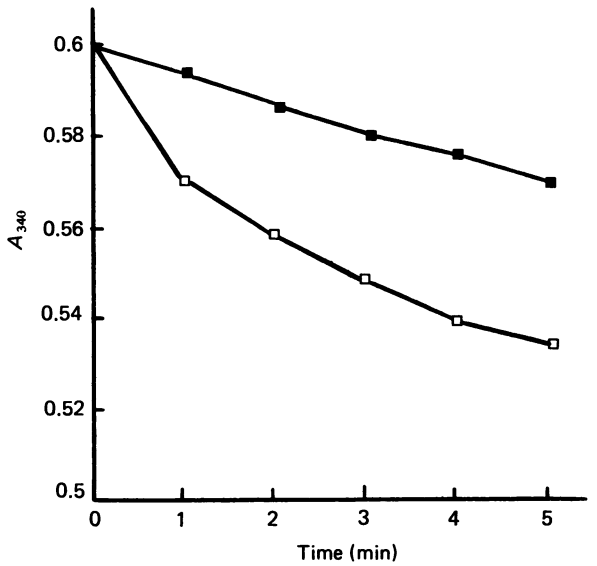


Fig. 2. Stimulation of NADPH oxidation by crocidolite fibres

Reactions were carried out as described in the Materials and methods section. The absorption of NADPH in the absence (■) or in the presence (□) of crocidolite was recorded at 340 nm. Each point represents the average of four experiments.

peroxidation in liver microsomes (Slater & Sawyer, 1971), gave MDA levels greater than but comparable with those observed with crocidolite (Fig. 1). The combination NADPH + chrysotile led also to an increase of MDA levels but chrysotile was less active than crocidolite (Table 1). Thus, the results presented here will mainly deal with crocidolite. The formation of MDA is dose-dependent: for example, 1 and 5 mg of crocidolite gave respectively one-fifth and one-half the MDA given by 10 mg after 5 min incubation (Table 1). Nevertheless, this shows that low amounts of fibres are still able to stimulate the NADPH-dependent lipid peroxidation. Table 1 also shows that preincubation of crocidolite with microsomes greatly affects the ability of the fibres to stimulate the NADPH-dependent lipid peroxidation. As shown in Table 2, lipid peroxidation induced by crocidolite and NADPH is not inhibited by superoxide dismutase, catalase or mannitol. Moreover, addition of 1 mM-desferrioxamine to microsomes incubated with NADPH and asbestos had no effect on MDA production during the first 5 min, but subsequently completely inhibited lipid peroxidation, since no more MDA was formed between 5 and 30 min (Fig. 1). One should note that desferrioxamine, a very efficient iron chelator, inhibited almost completely the lipid peroxidation induced by NADPH alone (Table 2).

NADPH oxidation is stimulated by crocidolite fibres

The rate of NADPH oxidation in rat liver microsomes was increased immediately after addition of crocidolite fibres (Fig. 2). This effect was observed only during the first few minutes, since after about 4 min NADPH was oxidized, in the presence of crocidolite, at roughly the same rate as it was in the absence of the fibres.

Lipid peroxidation induced by iron oxides

Since it was suggested that lipid peroxidation induced by asbestos fibres was related to the iron content of these minerals (Weitzman & Weitberg, 1985; Gulumian *et al.*, 1983), it seemed interesting to check the ability of some solid iron compounds to induce lipid peroxidation in rat liver microsomes. Magnetite (Fe_3O_4), containing both Fe^{2+} and Fe^{3+}, goethite ($FeOOH\alpha$) and haematite (Fe_2O_3) containing only Fe^{3+} were chosen. Moreover, magnetite particles are present as impurities weakly bound to the surface of chrysotile fibres. The results are shown in Table 3. Haematite and goethite were unable alone to induce lipid peroxidation and did not have any effect on the NADPH-dependent lipid peroxidation. On the contrary, the combination magnetite + NADPH led

Table 3. Lipid peroxidation in rat liver microsomes induced by iron oxides

Reaction conditions were the same as for Table 1 except the protein concentration (1 mg of protein/ml). Each value is the mean ± S.D. for four to six observations.

	MDA produced (nmol/mg of protein)
Control	0
+ NADPH	0.38 ± 0.12
+ Magnetite (5 mg)	0.30 ± 0.15
+ Haematite (5 mg)	0.03 ± 0.02
+ Goethite (5 mg)	0.04 ± 0.02
+ NADPH + magnetite (5 mg)	6.87 ± 0.75
+ NADPH + magnetite (1 mg)	2.62 ± 0.25
+ NADPH + haematite (5 mg)	0.31 ± 0.15
+ NADPH + goethite (5 mg)	0.35 ± 0.15

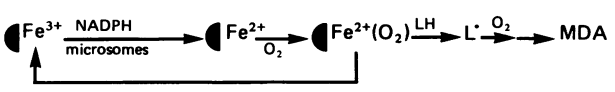
to a great and very rapid increase of MDA formation compared with the moderate formation of MDA induced either by NADPH alone or magnetite alone.

DISCUSSION

Asbestos fibres induced a moderate lipid peroxidation in the absence of NADPH, as previously described (Gulumian *et al.*, 1983; Weitzman & Weitberg, 1985). This indicates that these fibres, which present redox-active sites on their surface (Bonneau & Pezerat, 1983), are able to reduce and activate dioxygen into a species initiating lipid peroxidation. Also, as expected (Hochstein & Ernster, 1963; Aust *et al.*, 1985), a moderate lipid peroxidation is observed upon addition of NADPH to rat liver microsomes. This is due to the presence of iron ions in the microsomal solution (Aust & Svingen, 1982; Aust *et al.*, 1985), since MDA formation is completely inhibited by desferrioxamine, a very strong iron chelator (Morehouse *et al.*, 1984). Our results clearly show that lipid peroxidation in rat liver microsomes is greatly enhanced upon addition of both NADPH and asbestos fibres, crocidolite being more potent than chrysotile. This synergic effect cannot be due to a decomposition of H_2O_2 derived from the NADPH-alone-dependent enzymic reduction of O_2 (Kuthan *et al.*, 1978) to hydroxyl radicals catalysed by the fibres (Weitzman & Gracefa, 1984), since it is not inhibited by catalase. More likely, this synergic effect could be explained by an efficient electron transfer taking place from NADPH to the fibres, maybe partly via the NADPH:cytochrome *P*-450 reductase (Morehouse *et al.*, 1984; Pederson *et al.*, 1973), leading to an increase or a regeneration of the reducing sites at the surface of the fibres. This is supported by the observed effect of crocidolite on the rate of NADPH oxidation (Fig. 2). Our results indicate that this electron transfer is not mediated by superoxide anions and occurs only during the first minutes (Fig. 2), in agreement with the high rate of MDA production during the first 5 min and its decrease between 5 and 30 min (Fig. 1) to a level similar to that observed when microsomal suspensions contained only NADPH. It is also in agreement with the complete inhibition of MDA formation by desferrioxamine between 5 and 30 min (Fig. 1), indicating that after about 5 min lipid peroxidation is only dependent on NADPH. The nature of the redox-active sites is not certain, but since ferrous ions have a marked effect on lipid peroxidation (Aust & Svingen, 1982; Aust *et al.*, 1985), it is likely that iron present in asbestos either as an integral part of the silicate crystal structure as in crocidolite ($Na_2O,Fe_2O_3,FeO,8SiO_2,H_2O$), or as a contaminant at the surface of the fibres, as in chrysotile ($3MgO,2SiO_2,2H_2O$), could play this role. This is supported by the ability of magnetite, an impurity present on the surface of chrysotile, to induce a strong lipid peroxidation in liver microsomes and by the good correlation between the oxidation state of the iron ions in the different iron compounds studied and their capacity to induce lipid peroxidation. Actually, haematite and goethite, the ferric oxides, are unable to initiate lipid peroxidation or stimulate the NADPH-dependent lipid peroxidation, as opposed to crocidolite and magnetite, containing both Fe^{2+} and Fe^{3+} (Table 3). The absence of an inhibitory effect of desferrioxamine on the lipid peroxidation induced by NADPH and crocidolite together, during the first 5 min, indicates that the observed synergic effect of NADPH and crocidolite on lipid peroxidation is not due to an increase of free iron impurities in solution released from the fibres but also that the active iron ions inside the crocidolite structure are not readily accessible to desferrioxamine and cannot be efficiently chelated.

In Scheme 1 is presented a possible sequence of reactions leading to MDA. It is proposed that the initiator of lipid peroxidation is not hydroxyl radicals, derived from an asbestos-catalysed Haber–Weiss reaction, since no inhibition by superoxide dismutase or catalase has been observed. This is consistent with the absence of inhibitory effects of mannitol, a well-known scavenger of hydroxyl radicals. A ferrous–dioxygen complex is more likely, as proposed by Aust for lipid peroxidation induced by simple iron complexes such as Fe(ADP) (Aust & Svingen, 1982). Scheme 1 does not indicate another possible role of asbestos fibres, their involvement in the propagation step as catalysts of decomposition of lipid hydroperoxides into alkoxy radicals. The reason why the first reaction in Scheme 1, corresponding to the possible regeneration of the ferrous active sites of the fibres, is stopped after a few minutes is not clear. It is possible that, after a few minutes incubation, asbestos, which has been shown to adsorb large amounts of microsomal proteins (Gulumian *et al.*, 1983), is coated with proteins and lipids, preventing electron transfer. The observed inhibitory effects of asbestos preincubation on lipid peroxidation (Table 1) are consistent with this interpretation. Finally, in the absence of NADPH, the regenerating reaction does not occur but asbestos, as well as magnetite, contain enough active Fe^{2+} to initiate a moderate lipid peroxidation. But, since the active sites cannot be regenerated, the reaction stops rapidly (Gulumian *et al.*, 1983).

The ability of asbestos fibres to increase greatly, in association with NADPH, the microsomal lipid peroxidation gives a molecular basis for the previously observed membrane damage induced by asbestos and could explain its previously proposed role as a tumour promoter. Moreover, this study shows that this oxidative stress could not be prevented by the classical use of superoxide dismutase, catalase or iron chelators, pointing to the importance of understanding the molecular basis of asbestos fibre reactivity if one wants to control its toxic effects. Also, the strong lipid peroxidation induced by magnetite could be one of the origins of the excess incidence of lung cancers observed in some iron mines (Pham *et al.*, 1983).



Scheme 1. Possible sequence of reactions leading to MDA production

Symbols: LH, lipid; ◖, asbestos.

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