Silica earth provoked lung fibrosis with stimulation of lysosomal enzymes and lipid peroxidation in rats

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ABSTRACT The dynamics of the biological response of pulmonary tissue to silica dust (silica earth from Piotrowice, Poland, recommended as a domestic reference fibrogenic standard) was studied in rats after single-shot intratracheal instillation of a suspension of 20 mg of the dust for one, three, and seven months. Silica dust provoked pronounced pulmonary fibrosis as inferred from increased collagen content together with pathomorphological alteration (silicotic nodules). The lung burden of silica dust affected the lysosomal subfraction as manifested by an increase in its protein content with concomitant stimulation (release and presumably induction) of β -glucuronidase and cathepsin D and a transient (up to three months) stimulation of lipid peroxidation. Stimulation of activity of lysosomal enzymes and lipid peroxidation mediated by silica dust may reflect destructive metabolic processes resulting in the development of pulmonary fibrosis as the sign of a pathological repair mechanism. The extent of the effects brought about by silica earth testify that it may be recommended as a reference standard for evaluating the potential health hazard from industrial exposure to dusts containing SiO₂.

Occupational exposure to dust containing SiO₂ may lead to the development of silicosis.¹ The interaction of the dust particles with macrophages (paralleled by phagocytosis) has been postulated to be the critical factor in the action of silica on pulmonary tissues. This results in release of the macrophage fibrogenic factor stimulating fibroblasts to the overproduction of collagen and thus initiating a fibrotic process.²

On the other hand, the sequence of events in the process of phagocytosis of silica dust by macrophages involves the formation of secondary lysosomes (phagolysosomes) and the chemical interaction of SiO₂ with lysosomal membranes leading directly to rupture of the membranes, release of their hydrolytic enzymes, and, finally, cell death.³⁻⁵

In vitro experiments with cultures of alveolar macrophages and isolated red blood cells testing the release of the hydrolytic cytoplasmic and lysosomal enzymes into cellular medium allows one to assume that the interaction of SiO₂ with phagolysosomal membranes plays a crucial part in the SiO₂ provoked detrimental processes in the lungs.^{6,7}

It is also possible, however, that destabilisation of the membrane through the interaction with SiO₂ may

contribute to the concomitant enhancement of lipid peroxidation as may be inferred from direct in vitro assays with red blood cells and macrophages⁸⁻¹¹ and in vivo tests on rats with a short term lung burden of silica DQ-12.¹²

Thus both phenomena, lysosomal enzymes release, and lipid peroxidation enhancement may influence propagation of destructive processes in the membrane in the lung.

The present investigation was undertaken to determine whether in vivo, long lasting pulmonary deposition of silica dust (silica earth from Piotrowice, Poland, recommended as a domestic reference fibrogenic standard) affects lysosomal enzyme activity and the lipid peroxidation rate as compared with the fibrotic response of the lungs. Study of the dynamics and extent of these changes may be useful for the comparative estimation of the potential biological aggressiveness of SiO₂ containing dusts and contribute to the elucidation of the underlying mechanism of its harmful effects.

Materials and methods

ANIMALS AND TREATMENT

Experiments were carried out on male 200-250 g Wistar rats fed a laboratory food, Murigran

(Bioveterinary Industries, Gorzów Wlkp, Poland) with tap water ad libitum.

The rats were exposed by intratracheal instillation to silica earth from Piotrowice in Poland (78.6% of SiO_2 , av 96% of respirable fraction¹³) recommended as a domestic reference fibrogenic standard for SiO_2 containing industrial dusts.^{14,15} Controls were exposed in a similar manner either to rutile (TiO_2 crystal lattice), an inert respirable dust,^{13,16} or to physiological saline.

A suspension of 20 mg of the appropriate dust in 0.3 ml saline or 0.3 ml saline only was sprinkled, under anaesthesia with a drop of 4% ksylocain, directly on to the glottis. The animals were killed at one, three, and seven months after dosing and their lungs were used for further tests.

BIOCHEMICAL ASSAYS

The rats were beheaded under a light ether anaesthesia and the lungs were excised, washed with physiological saline, blotted on a filter paper, and immediately processed. The lungs were weighed directly and the dry weight was determined after initial five hour desiccation at 50°C and then, to a constant weight, over phosphorus pentoxide.

Hydroxyproline in the collagen fraction of dry lungs was determined colorimetrically after extraction procedure at 550 nm¹⁷ and converted to collagen by using the factor 7.46.¹⁸

The lysosomal fraction was extracted using the method of Sawant *et al.*¹⁹ A 10% (w/v) homogenate of fresh lungs in 0.25 M sucrose—0.01 M Tris-HCl, pH 7.4 obtained with Teflon glass Potter-Elvehjem homogeniser and squeezed through a bolting cloth (pores of 200 μm in diameter), was successively centrifuged at 4°C at 900 g for 10 minutes, 7000 g for 15 minutes, and 20 400 g for 35 minutes to sediment the nuclei, mitochondria, and lysosomes, respectively. After washing with 1.15% KCl and recentrifugation at 20 400 g for 35 minutes, the lysosomal fraction was suspended by rehomogenisation in 1.15% KCl. The protein content was determined according to Lowry *et al.*²⁰

Activity of β -glucuronidase (EC 3.2.1.31) was assayed with p-nitrophenol glucuronide as substrate; the p-nitrophenol formed was determined colorimetrically at 400 nm.²¹

Activity of cathepsin D (EC 3.4.23.5) was assayed with denaturated haemoglobin as substrate²²; the acid soluble products of hydrolysis of the protein was determined with Folin phenol reagent.²⁰

Lipid peroxidation in the homogenates and lysosomal membranes was evaluated on the basis of the detection of thiobarbituric acid reactive substances.²³ An extinction coefficient of 156 000 $\text{M}^{-1}/\text{cm}^{19}$ was used for quantifying malondialdehyde.²⁴

The results were statistically evaluated using Student's *t* test.

PATHOMORPHOLOGICAL EXAMINATION OF THE LUNGS

The lungs were fixed in situ at full inflation by the infusion of 10% neutral buffered formalin through the larynx and trachea. After ligation of the trachea all the thoracic organs were excised and placed in 10% buffered formalin solution for continued fixation. The sections were then gradually dehydrated in isopropanol (50–96%), cleared in xylene, and impregnated with paraffin. Haematoxylin and eosin stained paraffin sections were examined with a light microscope.

Results

The biological response of the rat's pulmonary tissue to a silica earth dust burden lasting for seven months is presented in table 1. One month after intratracheal instillation both the fresh and dry weights of the lungs had increased twofold and continued for the next six months. This change resulted from the development of pulmonary fibrosis since the collagen content of the lungs increased simultaneously. The lysosomal protein content, an index of the phagolysosomal response in silica laden cells (macrophages) increased concurrently.

These observations were confirmed by pathomorphological examination of the lungs during the course of exposure to silica earth dust (figs 1–5). Figure 1 shows a fragment of lung tissue of a control, saline treated rat with a normal pulmonary parenchyma and normal alveoli and bronchi. In fig 2 a fragment of lung tissue from a rat treated with rutile is shown: black dots represent rutile particles deposited in the walls of the alveolar ducts and in the cytoplasm of phagocytes in the lumen of the alveoli. No signs of progressive, reactive changes were observed. In the lungs from rats exposed to silica numerous silicotic nodules with strands of fibrous connective tissue were seen (fig 3) and as the exposure was continued, cytolysis of alveolar macrophages with thickening of alveolar septa in the periphery of nodules occurred (fig 4). At the end of seven months exposure pronounced fibrosis developed, resulting in the reduction of the pulmonary air spaces, glazing of the connective tissue, and focal cell disintegration. Thus the silica penetrating deeply into the lung had exerted a deleterious fibrogenic effect at its target sites.

The biochemical findings in the lysosomal subfraction of the pulmonary cells of rats exposed to silica, compared with the controls, also showed the damaging effects of the dust on the cell membrane as deduced from the increase (release) in the activity of the

Table 1 Increase in lung weight and pulmonary content of collagen and lysosomal proteins in rats after intratracheal instillation of silica earth

Treatment	Period of exposure (months)	Body weight (g)	Lungs		Collagen* (mg per lungs dry weight)	Lysosomal proteins† (mg per lungs fresh weight)
			Fresh weight (g)	Dry weight (g)		
Saline	1	259 ± 6	1.52 ± 0.05	0.26 ± 0.02	28.87 ± 1.30	6.23 ± 0.30
Rutile		245 ± 8	1.58 ± 0.07	0.24 ± 0.01	30.29 ± 1.47	6.48 ± 0.63
Silica earth		238 ± 7‡	3.16 ± 0.20§	0.55 ± 0.05§	69.75 ± 7.62§	13.90 ± 0.95§
Saline	3	324 ± 9	1.88 ± 0.18	0.26 ± 0.02	30.81 ± 2.27	8.08 ± 0.38
Rutile		330 ± 9	1.75 ± 0.08	0.35 ± 0.03	42.60 ± 1.98‡	8.05 ± 0.35
Silica earth		267 ± 11§	4.02 ± 0.33§	0.68 ± 0.08§	97.43 ± 10.15§	17.69 ± 0.80§
Saline	7	351 ± 13	1.97 ± 0.08	0.32 ± 0.01	38.64 ± 1.75	8.27 ± 0.39
Rutile		383 ± 15	2.14 ± 0.08	0.32 ± 0.02	41.78 ± 2.68	10.91 ± 0.86
Silica earth		343 ± 13	3.82 ± 0.19§	0.58 ± 0.05§	79.60 ± 6.86§	18.34 ± 0.76§

The rats were dosed intratracheally with (1) 20 mg of silica earth in 0.3 ml of saline and (2) reference controls with 20 mg rutile (TiO₂ crystal lattice) suspended in 0.3 ml saline or 0.3 ml saline only.
Results are the means ± SE for at least 12 rats.
*Hydroxyproline was converted to collagen by using a factor 7.46.¹⁸
†Determined according to Lowry *et al.*²⁰
|| ‡ § significantly different from concurrent control at p < 0.05, 0.01, and 0.001.

Fig 1 Lung of control rat, three months after intratracheal instillation of 0.3 ml physiological saline. Normal structure of pulmonary parenchyma with regular air space of alveoli (a), smooth contour of bronchi (b), and blood vessels (v) are seen. (Haematoxylin and eosin × 95.)

Fig 2 Lung of control rat three months after single intratracheal instillation of suspension of 20 mg rutile dust. Rutile (deep black) is seen deposited in wall of alveoli ducts and in cytoplasm of phagocytes (arrow) in lumen of alveoli. (Haematoxylin and eosin $\times 95$.)

Fig 3 Lung of rat three months after intratracheal instillation of suspension of 20 mg silica earth dust. In vicinity of bronchi (b) two silicotic nodules (n) with strands of fibrous connective tissue (c) are seen. (Haematoxylin and eosin $\times 95$.)

Fig 4 Lung of rat three months after intratracheal instillation of 20 mg silica earth dust. In vicinity of silicotic nodule (n) cytolysis of alveolar macrophages (m) is seen. Note thickening of alveolar septa in periphery of nodule (arrows). (Haematoxylin and eosin $\times 95$.)

Fig 5 Lung of rat seven months after intratracheal instillation of silica earth. Fragment of silicotic nodule with numerous fibroblasts (f) surrounded by bundles of collagen (c) and some multinuclear giant cells (g). (Haematoxylin and eosin $\times 240$.)

Table 2 Activity of lysosomal enzymes and lipid peroxidation in the lungs of rats with long lasting pulmonary deposition of silica earth

Treatment	Period of exposure (months)	Activity of β -glucuronidase (nmoles of p-nitrophenol formed per mg protein per 15 min)		Activity of cathepsin D (μ g of hemoglobin hydrolysed per mg protein per 15 min)		Lipid peroxidation (nmoles of malondialdehyde per mg protein)	
		Homogenates	Lysosomes	Homogenates	Lysosomes	Homogenates	Lysosomes
Saline	1	52 $\pm$ 5	60 $\pm$ 10	273 $\pm$ 26	400 $\pm$ 50	0.77 $\pm$ 0.05	3.35 $\pm$ 0.40
Rutile		81 $\pm$ 7 $\ddagger$	70 $\pm$ 20	310 $\pm$ 24	450 $\pm$ 50	0.82 $\pm$ 0.05	3.47 $\pm$ 0.52
Silica earth		265 $\pm$ 30 $\S$	230 $\pm$ 30 $\S$	625 $\pm$ 60 $\S$	630 $\pm$ 40 $\ddagger$	0.84 $\pm$ 0.07	4.03 $\pm$ 0.58
Saline	3	68 $\pm$ 3	70 $\pm$ 10	261 $\pm$ 18	510 $\pm$ 90	0.68 $\pm$ 0.01	3.25 $\pm$ 0.24
Rutile		61 $\pm$ 5	70 $\pm$ 10	271 $\pm$ 29	470 $\pm$ 30	0.65 $\pm$ 0.04	4.12 $\pm$ 0.38
Silica earth		215 $\pm$ 19 $\S$	160 $\pm$ 20 $\S$	484 $\pm$ 38 $\S$	1000 $\pm$ 110 $\ddagger$	0.64 $\pm$ 0.03	5.13 $\pm$ 0.24 $\parallel$
Saline	7	66 $\pm$ 12	70 $\pm$ 10	279 $\pm$ 16	340 $\pm$ 40	0.63 $\pm$ 0.07	3.16 $\pm$ 0.30
Rutile		70 $\pm$ 5	70 $\pm$ 10	279 $\pm$ 17	350 $\pm$ 60	0.77 $\pm$ 0.09	3.11 $\pm$ 0.27
Silica earth		141 $\pm$ 12 $\S$	140 $\pm$ 10 $\ddagger$	580 $\pm$ 63 $\S$	1130 $\pm$ 130 $\S$	0.85 $\pm$ 0.09	3.91 $\pm$ 0.36

 $\parallel$ $\ddagger$ $\S$ Details as in table 1.

hydrolytic enzymes, β -glucuronidase, and cathepsin D and the transient stimulation of lipid peroxidation (table 2). The activity of β -glucuronidase both in the lysosomal subfraction and the whole homogenate was increased two or three times during the course of exposure but with a tendency for the effects to diminish with time. The activity of cathepsin D increased gradually during the seven months of exposure and was most pronounced in the lysosomal fraction. The rate of lipid peroxidation in the lysosomes was significantly increased only after three months of exposure, although it tended to exceed the initial value during the whole period of observation.

Discussion

The study has shown that silica earth from Piotrowice ledge (Poland) recommended as a reference domestic standard for testing the fibrogenic effects of mineral dusts (cf Więcek *et al*¹⁴ and Szendzikowski *et al*¹⁵) exerts a profound pulmonary effect in rats after its instillation by the trachea in a dose of 20 mg for seven months. The effects include the development of fibrosis with specific silicotic nodules (cf table 1 and figs 1–5) and is accompanied by a lysosomal (phagolysosomal) membrane response manifested by an enhancement of the activity of hydrolytic enzymes and a transient stimulation of lipid peroxidation (cf tables 1 and 2).

It may be postulated that the stimulation of the activity of lysosomal enzymes may be caused by two mechanisms: (1) release of the enzymes from the destabilised membranes due to a direct action of the silica^{4–6} or due to silica provoked lipid peroxidation^{11,25}; (2) induction of the synthesis of the enzymes as indicated by the increased protein content in the

lysosomes (cf table 1).

These results corroborate data previously reported from *in vivo*²⁶ and *in vitro* (isolated macrophages, fibroblasts) studies which show the stimulatory effects of SiO₂ containing dusts on collagen biosynthesis^{7,27} and on phagolysosomal membrane destabilisation due to changes of the architecture and fluidity of the membrane leading finally to the release of hydrolytic enzymes into the surrounding medium.^{3,6,28}

Lysosomes, including phagolysosomes, participate in the physiological turnover of cellular components and the scavenging of undegradable extraneous materials; the first function may be disturbed by absorbed mineral dusts^{1,6,7,29} and that is the case with silica earth. Among the enzymes released from the lysosomal membranes by silica, cathepsin D seems to disturb the turnover of cellular proteins predominantly by the hydrolysis of the intracellular proteins^{3,5,6} as compared with lysosomes (cf table 1), procollagen,³⁰ and glycoproteins, matrices for connective tissue.³¹ It seems that the disturbance in the relation between these degradation processes triggers a rebound "repair" mechanism through the formation of collagen and the accumulation of connective tissue, thus leading to fibrosis.

The dynamics of the development of silica earth provoked fibrosis, with signs of specific lysosomal membrane derangements, shows that the "single shot" model of exposure is a valid one for the experimental, *in vivo*, evaluation of potential biological activity. Since the biological aggressiveness of the silica earth studied extends to the level of the lysosomal subfraction of the lung cells, it may be advisable to recommend it as a reference standard in evaluating the potential health hazard from industrial exposure to mineral dusts.

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