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Measurements of enzymes of collagen synthesis in rats with experimental silicosis

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Summary. The levels of two enzymes of collagen biosynthesis namely lysyl oxidase and prolyl hydroxylase were measured in the lungs of rats 3 and 6 weeks after receiving a single intratracheal instillation of silica or sterile saline. Circulating levels of lysyl oxidase were also estimated. Significant increased lung-enzyme activities were observed in the silica-exposed rats at both time intervals. Plasma levels of lysyl oxidase were also found to be raised in the silica-exposed rats. These changes were not, however, accompanied by profound pathological alterations. These results demonstrate that after exposure to silica histologically detectable fibrosis is preceded by significant changes in the activities of enzymes associated with collagen synthesis.

Keywords: silicosis, collagen biosynthesis, prolyl hydroxylase, lysyl oxidase

It is well known that inhalation of a variety of inorganic dusts such as silica, asbestos and beryllium can result in the development of lung fibrosis. Although much effort has been expended in studying the biochemistry of fibrotic disease it has often proved difficult to relate histological observations to biochemical measurements. It has been reported for example that the collagen contents of lungs from individuals with pulmonary fibrosis were within normal limits (Fulmer *et al.* 1980). Recently, however, there have been reports that increased levels of some of the enzymes involved in collagen biosynthesis—lysyl oxidase (extracellular) and prolyl hydroxylase (intracellular)—have been detected in the lungs of animals exposed to bleomycin or cadmium chloride (Counts *et al.* 1981; Chichester *et al.* 1981). Other investigators have also reported changes in the tissue and blood levels of such enzymes in experimental hepatic fibrosis (Carter *et al.* 1982; Siegel *et al.* 1978). Such observations suggested that the measurement of enzymes of collagen biosynthesis could be used as indicators of early changes in dust-induced pulmonary fibrosis. The lung activities of lysyl oxidase and prolyl hydroxylase and circulatory levels of lysyl oxidase were therefore studied in rats exposed to a fibrogenic dust.

Materials and methods

Silica. The DQ12 standard sample of silica used in this study was obtained from the Institute of Occupational Medicine, Edinburgh. Before use it was sterilized by autoclaving dry at 121°C for 15 min.

Animals. The experimental animals were Fischer F334 rats originally obtained from Charles River Breeding Laboratories Inc. Wilmington, USA. They have been bred under barrier-maintained conditions and the colony subjected to regular histological and microbiological screening. There was no

evidence of pulmonary infection in the rats used in this investigation.

Treatment. Male rats (10 weeks old) were weighed and randomly allocated to silica-treated and control groups. The animals were anaesthetized with ether and 0.5 ml of a sterile suspension of silica in phosphate-buffered saline (40 mg/ml) was instilled by tracheotomy, a technique we have found to give more even deposition of particulates than does peroral instillation. Control animals received 0.5 ml of sterile phosphate-buffered saline (PBS).

At 3 and 6 weeks following treatment a group of control and treated animals were weighed, anaesthetized, the thorax opened and the rats bled out from the right ventricle using heparin as anticoagulent. The lungs were perfused with PBS, removed, sliced into pieces approximately 2 mm square and roughly divided into two samples. The individual samples were weighed and then stored in liquid nitrogen. The blood was centrifuged, the plasma removed and also stored in liquid nitrogen, Lungs from a second set of animals from the treated and control groups were also removed intact, inflated through the trachea with 1% neutral formalin and processed for histology. Tissues from the liver and spleen were also fixed for later processing. Sections of tissues (5 μm) were either stained with haematoxylin and eosin or for reticulin fibres (Gordon & Sweets 1936). The severity of fibrosis was estimated using the assessment of Belt & King (1945) as modified by Wagner (see Brown *et al.* 1983). The scale was: grade 1, loose reticulin fibrils with no collagen; grade 2, compact reticulin with or without a little collagen; grade 3, somewhat cellular but made up mostly of collagen; grade 4, wholly composed of collagen fibres and completely acellular; and grade 5, acellular, collagenous and confluent.

Preparation of substrates for lysyl oxidase and prolyl hydroxylase assay. The purified [^{3}H]-collagen substrate, labelled with DL [6^{3}H]-lysine, for estimation of lysyl oxidase activity was prepared from the calvaria of 14-day chick embryos as described by Royce *et al.* (1980). The substrate for the estimation of prolyl hydroxylase activity was prepared from the tendons or calvaria of 14-day chick embryos according to the method of Kivirikko & Myllyla (1982)

Assays. All the assays were carried out using amounts of lung supernatant or serum which had been shown to give a linear relationship between product formation and enzyme activity.

Lysyl oxidase. Lysyl oxidase activity in samples of lung and plasma were measured according to the method described by Royce *et al.* (1980) except the activity of the collagen substrate in these studies was 1×10^6 ct/min per vial and the reaction mixtures were incubated at 37°C for only 4 h. For each sample duplicate vials were used for experimental and control samples. The latter contained β-aminoproprionitrile (50 μg/ml) to inhibit lysyl oxidase activity. By subtracting the radioactivity released in the control samples (i.e. non-specific tritium release) from that in the experimental, the actual enzyme activity was estimated.

Prolyl hydroxylase. Prolyl hydroxylase activity was assayed in the lung tissue by determination of tritiated water as described by Kivirikko & Myllyla (1982) except that the assay mixture contained 2×10^5 ct/min of substrate. The enzyme control samples in this assay were treated with 2,2-dipyridyl (50 μg/ml) to inhibit hydroxylase activity.

Samples of tissue and plasma from five silica and three control animals were used for determination of lysyl oxidase and prolyl hydroxylase activity at each of the two time points of sampling.

Results

Enzyme activity

The activities of prolyl hydroxylase and lysyl oxidase in lung samples and lysyl oxidase in the plasma of the treated and control animals are shown in Tables 1, 2 and 3 respectively.

Lung prolyl hydroxylase activity was increased at both 3 and 6 weeks following silica treatment (Table 1). The difference between exposed and control values was significant ($P<0.05$) whether the activities were calculated as ct/min/g lung wet weight or as ct/min/lung. Lysyl oxidase activity was also elevated in the lungs of treated animals although the increase expressed as ct/min/g lung wet weight attained significance at only the 10% level (Table 2). Increases in lung lysyl oxidase were reflected in the high levels of plasma enzyme in the silica treated animals (Table 3).

During the course of the study there was an increase in the weight of lungs in the silica-exposed groups as compared to the control; however the difference did not reach significance.

Histopathology

At 3 weeks there were macrophages in the lumina and infiltration of the walls of the respiratory bronchioles in the lungs from silica-treated animals. These cellular deposits were diffuse and very little reticulin fibre was present. These lesions were consistent with those classified as grade 1 (see Fig. 1*a*). In the animals killed at 6 weeks the lesions, although still in the vicinity of the respiratory bronchioles, were now seen in the interstitium where they had become much more circumscribed and contained a discernable increase in reticulin fibres. These are classified as grade 2 nodules (see Fig. 1*b*).

Discussion

Because lung fibrosis involves the abnormal deposition and accumulation of collagen it seems probable that the rate of synthesis of the protein and thus the progression of the disease may be regulated by the activities of collagen synthesizing enzymes. In this investigation attempts have been made to study the relationship between the activities of two such enzymes and the development of pulmonary fibrosis resulting from silica exposure. The enzymes selected for study were prolyl hydroxylase, which catalyses the hydroxylation of prolyl residues and is necessary for stabilizing the triple helix, and lysyl oxidase which initiates covalent cross-linkage in collagen. Silica was used as the fibrogenic agent in this study since this dust has been used extensively in this laboratory to induce pulmonary fibrosis and the natural history of silicosis in the Fischer 334 strain has been well documented (for example see Brown *et al.* 1983).

Increased levels of lysyl oxidase and prolyl hydroxylase have been reported in the lungs of animals exposed to bleomycin (Counts *et al.* 1981) and cadmium chloride (Chichester *et al.* 1981). The results presented here show that increases in the levels of these enzymes also occurs in the lungs of rats with experimental silicosis. Enzyme levels in the lungs of the silica-treated animals 3 weeks after treatment were approximately double that of the control values. By 6 weeks 3- and 2.5-fold increases in lung prolyl hydroxylase and lysyl oxidase activities respectively were detected. These marked increases in enzyme activities, at both 3 and 6 weeks, suggests a rapid increase in the rate of collagen synthesis. Histological examination of the lungs indicated that these changes were however not accompanied, at these times, by observable accumulations of collagen. With the concentration of silica used here, excess collagen accumulation does not become apparent until about 10 weeks after treatment.

Counts *et al.* (1981) reported that in bleomycin-induced pulmonary fibrosis the increase in lysyl oxidase activity was greater and occurred earlier than the increase in prolyl hydroxylase. In this study the in-

Table 1. Effect of silica (DQ12) on lung prolyl hydroxylase activity

	3 weeks after treatment		6 weeks after treatment	
Treatment	Activity/g lung (ct/min × 10^3)	Total activity/lung (ct/min × 10^3)	Activity/g lung (ct/min × 10^3)	Total activity/lung (ct/min × 10^3)
Control	5.3±0.18	4.4±0.09	2.9±0.31	2.4±0.33
Silica	*6.9±0.26	*8.6±0.46	*5.7±0.67	*9.3±1.15

Data are presented as mean±SEM.
Activity is expressed as ct/min/g of lung wet weight and ct/min/lung.
*Significantly different from control at $P<0.05$.

Table 2. Effect of silica (DQ12) on lung lysyl oxidase activity

	3 weeks after treatment		6 weeks after treatment	
Treatment	Activity/g lung (ct/min × 10^3)	Total activity/lung (ct/min × 10^3)	Activity/g lung (ct/min × 10^3)	Total activity/lung (ct/min × 10^3)
Control	12.3±1.4	10.2±1.2	10.5±0.8	8.5±0.94
Silica	**16.2±1.0	*20.6±1.1	**12.7±1.2	*20.8±1.7

Data are present as mean±SEM.
Activity is expressed as ct/min/g lung wet weight and ct/min/lung.
* Significantly different from control at $P<0.05$.
** Significantly different from control at $P<0.10$.

Table 3. Effect of silica (DQ12) on plasma lysyl oxidase

	3 weeks after treatment		6 weeks after treatment	
Treatment	Activity/ml plasma (ct/min × 10^3)	Total activity/rat (ct/min × 10^3)	Activity/ml plasma (ct/min × 10^3)	Total activity/rat (ct/min × 10^3)
Control	0.03±0.01	0.16±0.07	0.09±0.07	0.49±0.4
Silica	*0.29±0.08	*2.45±0.64	*0.28±0.05	*2.50±0.41

Data are presented as mean±SEM.
Activity is expressed as ct/min/ml of plasma and ct/min/rat.
Values obtained in the plasma were converted to total activity per rat by assuming that the total blood volume was 7% of body weight and plasma accounted for half the blood volume (Siegel *et al.* 1978).
*Significantly different from control at $P<0.05$.

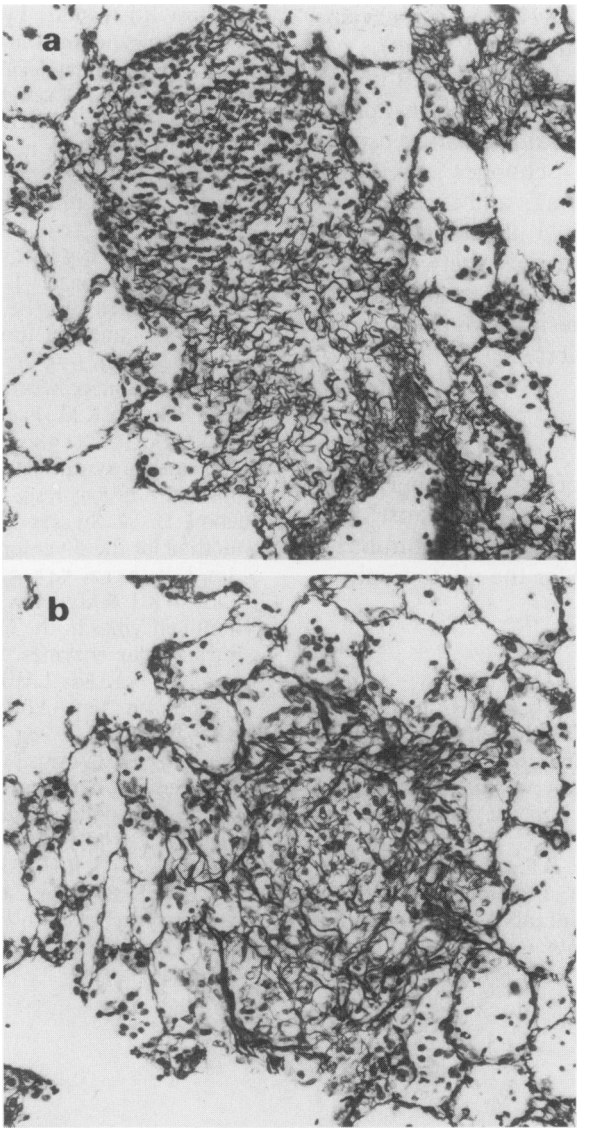

Fig. 1. Histological appearance of the lungs of animals 3 (*a*) and 6 (*b*) weeks after the intratracheal instillation of 20 mg silica. The sections were stained for reticulin by the method of Gordon & Sweets (1936). × 225.

creases in both enzymes occurred at the same time and were of similar magnitude; this is consistent with the report of Chichester *et al.* (1981) who found that after cadmium inhalation prolyl hydroxylase increases paralleled those of lysyl oxidase.

The increased tissue levels of lysyl oxidase measured in this study were accompanied by increases in plasma activity, the levels remaining constantly elevated over the exposure period. There are no other reports on plasma levels of lysyl oxidase in experimental lung damage; however animals with chemically induced liver injury show in-

creased circulating levels of this enzyme (Carter *et al.* 1982; Siegel *et al.* 1978).

The results of this study indicate that the measurement of enzymes such as prolyl hydroxylase and lysyl oxidase can be used to assess biochemical changes preceding observable tissue damage and repair. Using such methods it should also be possible to demonstrate relationships between biochemical measurements of collagen synthesis and the anatomical changes occurring in the lungs during fibrosis.

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