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INVOLVEMENT OF SUPEROXIDE IN THE PARAQUAT-INDUCED ENHANCEMENT OF LUNG COLLAGEN SYNTHESIS IN ORGAN CULTURE

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SUMMARY:

Exposure to paraquat *in vivo* results in increased synthesis and deposition of collagen. We examined collagen synthesis in organ cultures of neo-natal rat lungs in the presence of paraquat. Paraquat markedly increased the synthesis and accumulation of collagen in these cultures. This effect was abolished by superoxide dismutase. Our studies suggest that the mechanism of paraquat mediated increase in collagen synthesis may involve superoxide.

INTRODUCTION:

Paraquat (Methyl viologen, 1-1'-dimethyl-4,4'-bipyridinium dichloride) induces a rapid increase in lung collagen content in animals receiving the herbicide by inhalation, injection or orally (1-3). Superoxide anion (O_2^-) has been implicated in the toxicity of paraquat (4). Because of the interest in paraquat injury as a model for pulmonary fibrosis, it is of interest to examine the mechanism of paraquat-induced increases in lung collagen synthesis. Previous studies in our laboratory have shown the involvement of superoxide in collagen synthesis (5,6). In the studies reported here, we investigated the synthesis of collagen in lung organ cultures in the presence of paraquat, and the involvement of superoxide in this process.

MATERIALS AND METHODS:

Superoxide dismutase (2900 units/mg) was a product of Sigma and paraquat was purchased from Aldrich Chemical Company. L-[^{14}C]-U-proline, 291 mCi/mMole was obtained from New England Nuclear Corporation. The lung organ culture procedure has been described previously (7,8). The method consists in placing three or more 1 mm thick slices of neo-natal rat lung (Long-Evans) on Millipore filters (0.45μ pore size, 2.5 cm dia.) supported on 1ml Dulbecco-Vogt minimum essential medium, containing 10% fetal bovine serum, 50μg/ml ascorbate and antibiotics, in a Falcon plastic organ culture dish. Several such assemblies are placed in a gas tight chamber through which a humidified mixture of 5% CO_2 in air is circulated. Our previous studies showed that concentrations of O_2

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higher than in the ambient air also stimulated collagen synthesis (8). The rate of collagen synthesis was determined by measuring the incorporation of ^{14}C-proline as ^{14}C-hydroxyproline after pulse-labeling the tissue for 3 hours. Radioactive hydroxyproline assays were carried out by a published method (9) and the incorporated radioactivity was related to the total protein content measured as total ninhydrin-reactive material in the 6N HCl hydrolyzates of the tissues, expressed in leucine equivalents since leucine was used in standards (10). Total collagen content was determined on the basis of hydroxyproline assays (11), and DNA was measured in tissue homogenates by the diphenylamine procedure (12).

RESULTS AND DISCUSSION:

Previous studies have shown that exposure of humans and animals to paraquat, an herbicide, results in a rapid fibrotic response in the lung (1,13). Both the content and the synthesis (2,3) of collagen are markedly increased in lungs of animals exposed to paraquat. Paraquat administration also increased the levels of prolyl hydroxylase, a crucial enzyme in collagen synthesis, in the lungs of exposed animals (14,15). In order to examine if the enhanced synthesis and accumulation of collagen resulted from the direct effect of paraquat on lung cells, we measured the synthesis of collagen in organ cultures of neo-natal rat lungs. Paraquat was included in the culture medium for an initial 24 hour period and the tissues were then maintained in paraquat-free medium for an additional 48 hours. Preliminary experiments indicated that continuous exposure to paraquat after the initial 24 hours was not necessary to elicit a stimulatory response and exposures longer than 24 hours resulted in cell death, as evidenced by a decrease in the DNA content. Because of the isolation of these tissues from systemic responses such as circulating cells, proteins and immune mechanisms, the observed effects of paraquat may be ascribed to mechanisms inherent in lung cells.

As seen in Table I the collagen content of the cultures exposed to paraquat was consistently higher than in the control cultures. Maximal increase, over 50%, in collagen accumulation was observed in tissues exposed for 24 hours to 50μM paraquat and then maintained for an additional 48 hours in paraquat-free medium. Severe cytotoxicity and tissue necrosis was observed in tissues exposed to 100μM paraquat. The increased accumulation of collagen in organ cultures is consistent with *in vivo* effects (1-3).

TABLE I

COLLAGEN ACCUMULATION IN LUNG ORGAN CULTURES TREATED WITH PARAQUAT.

Additions	µg Hydroxyproline/mg DNA (mean ± SD)	Percent Increase
None	42.35 ± 5.23	
+Paraquat, 5µM	48.12 ± 4.56	14%
+Paraquat, 25µM	53.91 ± 6.12	27%
+Paraquat, 50µM	65.14 ± 6.48	54%

The exposure conditions and the assay procedures are described in the text and in the Methods section. DNA was measured in aliquots of the tissue homogenate. Each number represents the mean ± standard deviation of four determinations. Increases at 25 and 50 µM paraquat are statistically significant ($P < 0.005$). In lungs, collagen is the only protein containing significant amounts of hydroxyproline, therefore, the amount of hydroxyproline is a measure of lung collagen content.

In subsequent studies, the rate of collagen synthesis was examined in cultures exposed to paraquat (Table II). The cultures were exposed to 50µM paraquat for an initial 24 hours, and pulse-labeled with ^{14}C-proline at the end of the 48 hour culture period in paraquat-free medium. The overall incorporation of ^{14}C-proline was increased over 10% in the paraquat-treated cultures, however, the synthesis of ^{14}C-hydroxyproline was increased nearly 110%. A major part of the increase in total incorporation could be accounted for by the increase in collagen synthesis on the basis of ^{14}C-hydroxyproline. In experiments to be reported elsewhere, it was found that the synthesis of type III collagen chains in paraquat-treated cultures increased by 35% above control levels. These observations suggest that the stimulatory effect of low concentrations of paraquat on collagen synthesis may be quite specific and may not necessarily be related to non-specific chemical injury such as lipid peroxidation and generalized cell damage (16). Morphological studies on lung cultures exposed in this manner showed evidence of increased collagen accumulation but no significant tissue damage.

TABLE II
STIMULATION OF COLLAGEN SYNTHESIS IN LUNG ORGAN CULTURES BY PARAQUAT AND THE ABOLITION OF STIMULATORY EFFECT BY SUPEROXIDE DISMUTASE (SOD).

Additions	Total ^{14}C-proline incorporated (dpm/leucine equivalent x 10^{-4})	^{14}C-hydroxyproline formed (dpm/leucine equivalent x 10^{-2})
None	1.79	2.93
+Paraquat	1.99	6.13
+Paraquat+SOD(25 units/ml)	1.85	4.00
+Paraquat+SOD(50 units/ml)	1.94	2.77

Lung cultures were maintained in the presence of the additions shown in the table and were labelled with 3μCi of ^{14}C-proline for 3 hrs as described in the text. In cultures treated with SOD, the enzyme was present throughout the culture period. Total radioactivity incorporated as well as ^{14}C-hydroxyproline in the tissues were determined as described in the Method section. Each number represents the average of three determinations.

As in case of ozone and oxygen toxicity, paraquat toxicity has been attributed to the production of superoxide anions (17,18) by the reaction

$$PQ^{++} \underset{-e}{\overset{+e}{\rightleftharpoons}} PQ^{+\cdot}$$

$$PQ^{+\cdot} + O_2 \longrightarrow PQ^{++} + O_2^{\cdot-}$$

We examined the involvement of superoxide in the stimulation of collagen synthesis by paraquat. The cultures were exposed to paraquat in the presence of superoxide dismutase, an enzyme which specifically destroys the superoxide radical (19). As seen in Table II addition of 25 units/ml of superoxide dismutase, decreased the stimulatory effect from 110% to 35% and 50 units/ml abolished the stimulatory effect completely. Under these conditions the rate of collagen synthesis was the same as in controls. These data confirm the involvement of superoxide in the stimulation of collagen synthesis by paraquat. Superoxide dismutase has also been shown to protect against paraquat toxicity *in vivo* (17).

We have previously shown the involvement of superoxide as a stimulant for collagen synthesis (5,6). In these studies, early passage WI-38 cells were exposed to superoxide generated by the oxidation of medium ascorbate or generated photochemically, from riboflavin. Superoxide dismutase abolished the collagen stimulatory effect both of ascorbate and of riboflavin. The present studies lend further support to the concept that superoxide is a mediator of increased collagen synthesis. It may be speculated that the fibrogenic effects of paraquat, hyperoxia and ozone in lungs and the increase in collagen synthesis in inflammed tissues involves superoxide mechanisms, since superoxide is the common injurious species in these tissues.

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