The Effects of Exposure to Ozone on Collagen in Lungs and Skin

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INTRODUCTION

Studies of the toxicity of inhaled ozone, a major constituent of photochemical smog, have shown that long-term exposure to ozone at near-ambient concentrations results in morphologic changes in the pulmonary tract characterized by increased accumulation of connective tissue components in bronchioles and alveolar ducts¹⁻⁴. Biochemical studies have also shown the ozone-induced synthesis increased and that it is collagen which accumulates most in connective tissues^{4,5}. Such findings indicate that increases in the amount of collagen in the lungs may relate to pulmonary fibrosis and may indicate a chronic condition caused by ozone. Collagen isolated from various human and animal tissues has properties which depend on the age of the subject. For example, the solubility of dermal collagen varies with age, that is, the soluble collagen extractable from the skin decreases with age⁶⁻⁸. This indicates that the collagen has become more rigid and stable with the passage of time. Therefore, it can be expected that the collagen which has accumulated in the lungs due to exposure to ozone may affect collagen's overall solubility here.

The present study was undertaken to evaluate the effects of ozone exposure on lung collagen. Experiments were designed to investigate collagen level and its solubility in the lungs of mice and rats exposed to ozone. The collagen level was compared with that of reduced glutathione (GSH) which has been shown to be both a sensitive and quantitative indicator to monitor the effects of ozone⁹. Since collagen in the lungs of adult animals is almost insoluble either in a neutral salt or an acetic acid solution, both of which can extract collagen without denaturation^{10,11}, solubility was measured following the procedure described by Nimni et al.⁸ in which cysteamine solubilized significant amounts of insoluble collagen.

Solubility was also examined in rat dermal collagen to clarify the effects of ozone on skin as long-term exposure to nitrogen dioxide has previously been shown to induce increases in total and soluble collagen levels in the skin of guinea pigs¹².

MATERIALS AND METHODS

Two hundreds and twenty male 4-week-old ICR-Jcl mice and eighty SD-Jcl rats obtained from a breeding laboratory (CLEA Japan, Inc., Tokyo) were used. The animals were maintained on commercial chow and water *ad lib.* except during exposure periods. The experimental environment was controlled to a temperature of $22 \pm 2^\circ\text{C}$.

Groups of 120 mice each of 5-week-old were exposed to 0.2, 0.5, 1.0, and 1.7 ppm ozone 4 hours a day for 5 days or to 1.0 ppm ozone 4 hours a day for 10, 20, 30, or 40 days. Four-fifth animals subjected to the 40 days exposure were returned to pre-experimental conditions for 4, 10, 18, or 28 days. Groups of 40 rats were exposed to 1.0 ppm ozone 4 hours a day, 5 days a week, for 1, 3, 6, or 10 weeks. An equivalent chronological series of 10 each of control animals was kept under ambient conditions. A detailed description of the exposure procedure is given elsewhere⁹.

All mice were sacrificed by cervical dislocation and the lungs were removed, blotted, weighed, and homogenized in 8 ml of an ice-cold 0.02 M disodium ethylenediaminetetraacetate solution. For the GSH assay, 5 ml of homogenate was used with the remainder kept for the collagen assay. GSH was determined in the form of non-protein sulfhydryls following the method of Sedlak and Lindsay¹³. Independent determinations of GSH gave values which account for 95% of the non-protein sulfhydryls in

the homogenates¹⁴). Collagen was determined from the hydroxyproline content of acid hydrolyzates in the homogenates following Woessner¹⁵). In the presence of 6 N hydrochloric acid, 3 ml of homogenate was hydrolyzed for 3 hours at 130°C and dried in a vacuum at 70°C. The dried hydrolyzate was resuspended in 8 ml of water and filtered through filter paper. Aliquots of the filtrates were used to measure hydroxyproline which converts to its collagen equivalent multiplied by a factor of 7.46¹⁶). In this procedure, the elastin which inevitably coexists and contains about 2% hydroxyproline was not removed from the sample to be assayed. In the lungs, however, the amount of hydroxyproline derived from elastin is only about 6% of that derived from collagen¹⁷), and thus is not significant.

Rats were killed by cerebral concussion. The lungs and approximately 0.5 g of dorsal skin were removed, blotted, weighed, and lyophilized. The dried samples were used to estimate collagen solubility according to a slightly modified form of the method of Nimni et al.⁸) described below. Each sample was homogenized in 30 ml of ice water with a Polytron homogenizer until the sample was disintegrated. The homogenate was centrifuged at a temperature of 4°C at 40,000 g for 1 hour. The precipitate was homogenized in water and centrifuged again and finally homogenized in 30 ml of ice-cold 0.5 M sodium chloride solution. The resulting suspension was shaken overnight at 4°C and centrifuged a third time. The final supernatant solution was considered to represent the salt-soluble collagen fraction and the resulting precipitate was extracted overnight at 4°C with 30 ml of 0.5 M acetic acid solution in the case of the lung sample or 30 ml of 0.5 M citrate buffer solution (pH 3.6) in the case of skin. The acid-soluble collagen fraction was obtained after the centrifugation and the resulting precipitate was further extracted for 3 days at 4°C with 30 ml of 0.2 M cysteamine solution in 0.6 M sodium chloride (pH 7.0). The cysteamine-soluble and insoluble fractions were separated by centrifugation. Each supernatant fraction was concentrated in a collodion bag (Sartorius Membranfilter GmbH, Göttingen) and constituted the insoluble residue. The residues were washed with water several times and finally suspended in a small amount of water. The insoluble collagen fraction was washed with water once and was also suspended in 3 ml of water. The above suspensions were used to determine collagen as described above. About 90% of the total collagen was thus recovered.

RESULTS

Table 1 shows the effects of exposure to ozone for 5 days on GSH and collagen levels in mouse lungs. GSH levels increased linearly as a function of ozone concentration. GSH increased by 4.8 to 25.0% ($p < 0.05$ level of significance) following exposure to from 0.2 to 1.7 ppm ozone. The linear regression coefficient between ozone concentration and GSH was 0.997 ($p < 0.01$). On the other hand, there was no change in collagen following ozone exposure, although lung wet weights increased significantly at 1.0 ppm or more.

The effects of exposure to 1.0 ppm ozone for 0 to 40 days on the GSH and collagen levels in mouse lungs are shown in Figure 1. GSH levels increased significantly as a function of exposure duration up to 30 days and then decreased at 40 days although the levels remained significantly higher than those of the controls ($p < 0.01$). Collagen levels were steady at

Table 1 Effects of Five Days Exposure to Ozone on GSH and Collagen Levels in Mouse Lungs*

Ozone	n	GSH	Collagen
ppm		nmole/lung	mg/lung
0.0 (Controls)	20	421 ± 6	1.68 ± 0.04
0.2	10	441 ± 6	1.74 ± 0.06
0.5	10	452 ± 10	1.72 ± 0.06
1.0	10	484 ± 6	1.68 ± 0.06
1.7	10	526 ± 10	1.76 ± 0.06

* Data are expressed as the mean ± SE.

at the tenth day but then increased linearly as a function of exposure duration. At the fortieth day, the level was 26.0% higher than the control level ($p < 0.05$). Figure 2 shows GSH and collagen levels in the lungs of mice exposed to 1.0 ppm ozone for 40 days, which were then returned to pre-experimental conditions. The GSH had decreased sharply and was at the control level by the tenth day, whereas although the collagen level had also decreased, it was at a much slower rate. In fact, collagen levels remained elevated even 28 days after ozone had been discontinued (9.2% higher than controls, but not significant).

Fig. 1. Effects of repeated exposure to 1.0 ppm ozone on GSH and collagen levels in mouse lungs. Circles represent means and extended lines, SE's (n=10).

Fig. 2. GSH and collagen levels in mouse lungs following the end of the 40-day exposure to 1.0 ppm ozone. Circles represent means and extended lines, SE's (n=10).

Table 2 Collagen Levels and Percentage Distributions of the Different Fractions Extracted from the Lungs of Ozone-exposed and Control Rats*

Period of Exposure	Collagen Fractions				Total Collagen
	Salt Soluble	Acid Soluble	Cysteamine Soluble	Insoluble	
	mg/lung	mg/lung	mg/lung	mg/lung	mg/lung
1 week	0.05±0.01 (0.5)	0.06±0.00 (0.5)	0.03±0.01 (0.3)	10.2±0.5 (98.6)	10.3±0.5 (100)
Controls	0.05±0.00 (0.6)	0.05±0.01 (0.6)	0.02±0.01 (0.2)	9.0±0.7 (98.6)	9.2±0.7 (100)
3 weeks	0.04±0.01 (0.2)	0.07±0.01 (0.4)	0.05±0.01 (0.3)	16.3±0.8 (99.0)	16.5±0.8 (100)
Controls	0.03±0.01 (0.2)	0.06±0.01 (0.4)	0.03±0.01 (0.2)	14.6±0.9 (99.2)	14.7±0.9 (100)
6 weeks	0.03±0.00 (0.1)	0.08±0.01 (0.4)	0.07±0.01 (0.3)	19.1±0.9 (99.1)	19.2±0.9 (100)
Controls	0.02±0.00 (0.1)	0.06±0.01 (0.3)	0.07±0.01 (0.4)	17.3±1.3 (99.1)	17.4±1.3 (100)
10 weeks	0.02±0.01 (0.1)	0.10±0.01 (0.3)	0.02±0.00 (0.1)	29.4±1.9 (99.5)	29.6±1.9 (100)
Controls	0.02±0.01 (0.1)	0.07±0.02 (0.3)	0.02±0.00 (0.1)	21.3±1.4 (99.5)	21.4±1.4 (100)

* Data are expressed as the mean±SE (n=8-10). The parentheses show the percentage distributions.

Table 2 shows the effects of the levels and solubility of collagen from the lungs of rats exposed to 1.0 ppm ozone 1, 3, 6, and 10 weeks. Total collagen increased with age. About 99% of the collagen was insoluble in salt, acid, or cysteamine solutions, although the insoluble fraction tended to increase with as the age of the animal increased. Both acid-soluble and insoluble collagen increased with increasing exposure. In the lungs of rats exposed for 10 weeks, the acid-soluble fraction increased 46% ($p<0.01$) and the insoluble fraction 38% ($p<0.01$). However, the distribution between the fractions remained the same. Most of the lung collagen in 5-week-old mice was insoluble in salt, acid, or cysteamine solutions and the total soluble collagen was only 4%.

Table 3 Fractions of Collagen Extracted from the Skin of Rats Exposed to Ozone for 10 Weeks*

	Exposed	Control
	% of wet weight	% of wet weight
Salt Soluble	1.1±0.1	1.1±0.1
Acid Soluble	0.9±0.1	0.9±0.1
Cysteamine Soluble	5.3±0.2	5.3±0.2
Insoluble	13.2±0.8	12.5±0.7
Total	20.5±1.0	19.7±0.9

* Data are expressed as the mean±SE (n=10).

The levels and solubility of collagen from rat skin did not change even after 10 weeks exposure to 1.0 ppm ozone (Table 3). However, the moisture content of the skin decreased significantly, i. e. to 60.8±0.4% of wet weight in ozone-exposed rats as opposed to 62.2±0.2% in controls ($p<0.05$).

DISCUSSION

The results indicate that collagen levels in lungs increase following relatively long-term exposure to ozone. Short-term exposure increases GSH and presumably also the rate of collagen synthesis^{4,5}, but not the collagen levels themselves. Changes in lung collagen does not indicate the effects of ozone at early stages of exposure. In the long-term case, elevated collagen levels decrease more slowly than GSH after exposure is discontinued. Similar results have been demonstrated by Hussain et al.⁵ who found that an increase in the amount of collagen in the lungs of rats exposed continuously to 0.8 ppm ozone occurred later than an increase in its synthesis rate and that collagen level remained elevated for up to 28 days after the animals were returned to ambient filtered air. It has been suggested that because both the synthesis and degradation of collagen in lungs may occur at the early stages of exposure, collagen levels appear unchanged. During the next stage when metabolic activity increases to protect the organism against ozone's oxidative toxicity, GSH increases,⁹ the rate of collagen degradation decreases and thus the accumulation of collagen occurs. A possible structural change in lung collagen caused by ozone has been described by Buell et al.¹⁸.

Accumulated collagen remaining in the lungs for a long period may reflect irreversible pulmonary changes such as pulmonary fibrosis^{1,2}, and increased collagen may be a quantitative monitor of the chronic effects of ozone. Collagen, however, is not specific for ozone toxicity and has been reported in animals exposed to high as well as low concentrations of oxygen¹⁹, silica^{20,21}, and asbestos²². On the other hand, when animals are exposed to nitrogen dioxide, there is a decrease in collagen levels in the lungs^{12,17}. Such differences between the actions of ozone and nitrogen dioxide are important when considering their individual biological effects.

An attempt to solubilize the collagen in mouse and rat lungs by treatment with cysteamine which can solubilize significant amounts of insoluble dermal collagen was unsuccessful here. It has been reported that salt-soluble collagen is newly synthesized and becomes acid-soluble and cysteamine-soluble with the passage of time⁸. Bradley et al.¹⁰ have observed that less than 1-2% of the total collagen in adult rabbit lungs can be extracted with acetic acid and that even with the use of an inhibitor of the cross-linking of collagen molecules which increases the organic yield of soluble collagen, only 5% can be extracted from the lungs. This insolubility is believed to be due to covalent cross-links of tropocollagen and the association of collagen with proteoglycans and possibly with other proteins^{23,24}. The exposure to ozone made no difference on the solubility of collagen in the lungs, indicating the ozone-induced collagen and the original form are identical in this respect.

Collagen fibers are found in the tracheobronchial tree, pulmonary blood vessels, and alveolar interstitium²⁵⁻²⁷ and may play an important role in the lung's mechanical properties, however, it is still unclear to what extent accumulated collagen affects them. Bartlett et al.²⁸ have shown a decrease in lung tissue elasticity in rats exposed to 0.2 ppm ozone for 30 days and suggested that this may be caused by its effect on collagen. The study of elastin, another important component in pulmonary connective tissue and possibly a factor in lung mechanics^{24-26,29} is also necessary to further understand the effects of ozone.

Kleinerman¹⁷⁾ has demonstrated elastin and collagen degradation in hamsters exposed to nitrogen dioxide.

Skin which comes into direct contact with the ozone in air may be affected by its action. There is a loss of solubility when the soluble dermal collagen is exposed *in vitro* to the gaseous phase of tobacco smoke³⁰⁾. Soluble dermal collagen in guinea pigs exposed to nitrogen dioxide for 180 days has increased while insoluble collagen has decreased¹²⁾. In the present study, the amounts and solubility of dermal collagen in rats were unchanged even after the 10 weeks' exposure to 1.0 ppm ozone, although moisture content decreased. The period of exposure to ozone in this experiment may be insufficient to induce changes in the nature of dermal collagen. This loss of water from skin may be associated with the unthrifty appearance and coarseness of pelage observed in rabbits exposed to ozone for one year³¹⁾.

Despite suggestive evidence that ozone may accelerate the aging process³¹⁾, there is little information concerning ozone-induced biochemical changes related to aging. Solubility is perhaps the simplest measure of the age-related properties of collagen. The present experiments, however, were ineffective in assessing the effects of ozone. More research is needed to study the effects on collagen of prolonged ozone exposure.

SUMMARY

The effects of repeated exposure to ozone on the levels and solubility of collagen in mouse and rat lungs and in rat skin were studied. The lung collagen levels of mice exposed to 0.2 to 1.7 ppm ozone for 5 days remained unchanged, although GSH levels indicate the effects of ozone increased as a function of ozone concentration. A significant increase in lung collagen occurred in mice exposed to 1.0 ppm ozone for 40 days. Raised collagen levels decreased more slowly than GSH levels after animals were returned to the original pre-experimental conditions. Collagen in mouse and rat lungs was almost completely insoluble in 0.5 M sodium chloride, 0.5 M acetic acid, or 0.2 M cysteamine solutions. This insolubility continued when rats were exposed to 1.0 ppm ozone for up to 10 weeks. Amounts and solubility of collagen in rat skin were also unchanged following exposure to 1.0 ppm ozone for 10 weeks.

Acknowledgements : This work was supported in part by a grant from the Environmental Agency entitled "Studies of Biological Effects of Air Pollution" (1979-1980). Portions this paper were presented at the Fifty-first Annual Meeting of the Japanese Society for Hygiene (Sapporo, 1981) and the Twenty-second Annual Meeting of the Japan Society of Air Pollution (Akita, 1981).

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オゾン暴露の肺及び皮膚コラーゲンへの影響

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オゾン繰り返し暴露のマウス及びラット肺及びラット皮膚コラーゲンへの影響を検討した。0.2-1.7 ppm オゾン 5 日暴露を行ったマウス肺では、オゾンの影響の指標として用いられる還元型グルタチオン量は濃度に比例して増加したが、コラーゲン量は変化しなかった。マウス肺コラーゲン量の有意の増加は 1.0 ppm オゾン40日の暴露で起り、増加したコラーゲンは、暴露終了後還元型グルタチオンより緩やかに減少した。マウス及びラット肺コラーゲンの大部分は 0.5 M 塩化ナトリウム、0.5 M 酢酸、及び 0.2 M システアミン溶液に不溶で、その不溶解性は、ラットに 1.0 ppm オゾン10週まで

暴露しても変らなかった。また、1.0 ppm オゾン10週暴露したラット皮膚コラーゲンは、その溶解性及び量に変化が見られなかった。

(受付 1982年2月9日)