Vol. 83, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 28, 1978 Pages 392-397

ALTERATION OF COLLAGEN SYNTHESIS IN LUNG ORGAN CULTURES BY HYPEROXIC ENVIRONMENTS

Rajendra S. Bhatnagar, M. Zamirul Hussain,
Jerome A. Streifel, Maximita Tolentino and
Belma Enriquez

Laboratory of Connective Tissue Biochemistry
School of Dentistry
University of California, San Francisco, CA 94143

Received May 22,1978

SUMMARY:

Organ cultures of neo-natal rat lungs continue to accumulate collagen at a nearly linear rate for 5 days, when maintained in an atmosphere consisting of 5% CO_2 in air. Nearly 70% of the collagen synthesized in these cultures is Type I and 30%, Type III. When these cultures are maintained in 5% CO_2 + 95% O_2, the per cell accumulation of collagen increased. Although there is no significant change in the amount of radiolabeled proline incorporated, the rate of incorporation per cell is increased since there appear to be fewer cells in the O_2-treated cultures. These parameters of collagen synthesis parallel lung O_2 injury *in vivo*. There is a shift in the type of collagen synthesized, nearly 70% being Type III and 30%, Type I in O_2-exposed cultures. This finding is reminiscent of tissue repair after injury. Such changes in collagen may contribute to pulmonary connective tissue disorders in oxidant-induced lung injury.

INTRODUCTION:

Connective tissue alterations are a major aspect of lung disorders. Since collagen is a major protein component of lung connective tissue, qualitative and quantitative changes in collagen have been implicated in pulmonary dysfunction caused by a variety of etiologic agents (1). It is important to determine the molecular mechanisms involved in the development of such changes. Because injured lungs *in vivo* are subject to a variety of systemic influences including circulating cells and proteins, and immune phenomena, a clear cut delineation of mechanisms involved in lung collagen alteration is not possible. We have developed a simple organ culture procedure using neo-natal rat lung slices, which mimics many biochemical characteristics of lungs *in vivo* (2,3). Using this model we have investigated the alterations in collagen synthesis and accumulation caused by an oxidant atmosphere, consisting

0006-291X/78/0832-0392$01.00/0

of 95% O_2. Our studies show increased collagen accumulation and altered synthesis of Type I and Type III collagen chains under these conditions.

MATERIALS AND METHODS:

Lung organ cultures were prepared as described elsewhere (2,3). The method consists in placing 1mm thick slices of neo-natal rat lung (Long-Evans) on millipore filters (0.3μ) supported on 1ml Dulbecco-Vogt minimum essential medium in an organ culture dish. Several dishes were placed in a humidified, gas tight chamber in which appropriate gas mixtures were circulated. The collagen content of cultured lung tissues was measured by assaying for hydroxyproline (4). The rate of collagen synthesis was determined by measuring the incorporation of 3,4-[$^3H_2$]-L-proline (New England Nuclear, 25 Ci/mMole) into nondialyzable ^3H-hydroxyproline after pulse labeling for 3 hours at different times in culture (5). The DNA content of lung organ cultures was determined by the diphenylamine procedure (6), and the total protein content of the cultures was assayed by measuring the total ninhydrin-reactive material in 6N HCl hydrolyzates, (7). The labeled collagens synthesized in the presence of β-aminopropionitrile (8) were extracted after homogenization of tissues followed by pepsin digestion (9). Separation of Type I and Type III collagen chains was carried out by affinity procedures using thiol-activated sepharose columns to retard the cysteine-containing Type III chains (10).

RESULTS AND DISCUSSION:

Previous studies have shown that when animals are exposed to high O_2 atmospheres, the total DNA content in the lungs is decreased and the lungs exhibit morphological evidence for fibrosis, as an apparent increase in collagen deposition (11).

Organ cultures of neo-natal rat lungs maintained in an atmosphere containing 95% air + 5% CO_2, continue to accumulate DNA and collagen at a nearly linear rate for five days or longer (2,3). In order to determine if exposure to high O_2 atmospheres elicited a response in organ cultures, similar to their response *in vivo*, cultures were maintained in an atmosphere consisting of 95% O_2 + 5% CO_2. As seen in Fig. 1, the DNA content of cultures exposed to O_2 was markedly decreased. Under these conditions, there was an increase in the accumulation of collagen per cell measured as a ratio of collagen content to the DNA content of the cultures (Fig. 2). Similar observations on collagen accumulation have been reported in lungs of animals exposed to high O_2 atmospheres. Electron microscopic studies of O_2-exposed cultures, indicated that there was a thickening and obliteration of interalveolar septa, with the appearance of loosely packed fibrous collagen masses in the inter-

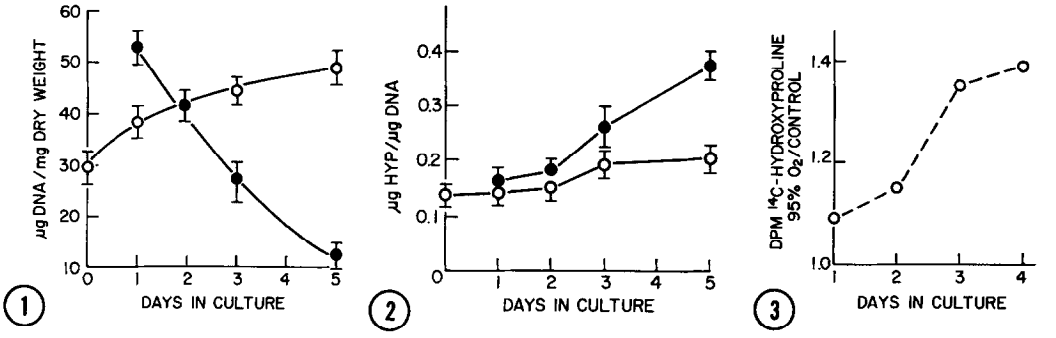

Fig. 1. DNA content of Lung Organ Cultures at different times in the presence of 95% Oxygen.

Procedures for the culture and DNA analysis are described in the Methods section. The dry weight was determined after lyophilizing the tissues to a constant weight. Each point represents the mean of five determinations and error bars show the standard error of the mean. O–O, Control; ●–●, 95% O_2.

Fig. 2. Hydroxyproline content of Lung Organ Cultures at different times in the presence of 95% Oxygen.

Procedures for the culture, analyses and statistical conventions are as described in the Legend to Figure 1. Each point represents the mean of five determinations. ●–●, Control; O–O, 95% O_2.

Fig. 3. Rate of Synthesis of Collagen in Lung Organ Cultures at different times in the presence of 95% Oxygen.

Procedures for the culture and analyses are as described in the Methods section. Collagen synthesis is indicated as a ratio of ^{14}C-hydroxyproline DPM formed per µg DNA in cultures grown in 95% O_2 to those grown in 20% O_2 (control).

stitial space. In this respect these cultures exhibited morphological characteristics reminiscent of lungs made fibrotic *in vivo*. These studies will be published elsewhere. Increased accumulation of collagen in lungs of animals exposed to high O_2 tensions has been correlated with increased protein synthetic activity (12,13). In order to determine if increased collagen accumulation per cell resulted from increased synthesis of collagen in cultures exposed to 95% O_2, the rate of synthesis of collagen was determined as the synthesis of ^{3}H-hydroxyproline from ^{3}H proline, administered in 3 hour pulses,

TABLE I

COLLAGEN CHAIN TYPES SYNTHESIZED IN LUNG ORGAN CULTURES IN THE PRESENCE OF 95% OXYGEN

	Relative Proportions of Pulse-Labelled Collagen(%)	
	Type I	Type III
20% Oxygen	69	31
	71	29
95% Oxygen	33	67
	38	62

Lung cultures were grown in gas mixtures containing 20% O_2 (Control) and 95% O_2 for 24 hours and were then labelled with 10μCi/ml of 3,4-[^{3}H]-L-proline in the presence of 100 μg/ml 3-aminopropionitrile for an additional 48 hours. Collagen was extracted (10 culture plates) and chromatographed as described in the Methods section.

after varying exposures to the hyperoxic environment. The data from this experiment (Fig. 3) showed a marked increase in the synthesis of ^{3}H-hydroxyproline per cell, on the basis of the DNA content of the cultures. These data substantiate observations *in vivo* (13,14) and explain the increased accumulation of collagen in lungs injured by hyperoxic environments.

Normal lungs parenchyma contains approximately 70% Type I collagen and nearly 30% Type III collagen (9). The proportions of the different collagen chains are altered in human idiopathic pulmonary fibrosis (9). We have examined chain composition of control and O_2-exposed lung organ cultures to determine, if the rates of syntheses of these distinct gene products were altered. As seen in Table I, in control cultures, nearly 70% of the total extractable labeled collagen was Type I, and 30%, Type III. These proportions were reversed in cultures maintained in the 95% O_2 + 5% CO_2 environment. Previous studies have shown that in fibrotic human lungs, the proportion of Type III collagen is significantly decreased (9). These studies reflect the long term accumulation of collagen in lungs presumably as a result of com-

pleted repair in lungs by unknown causes. Studies on collagen chains in tissues of different ages have shown that Type III collagen predominates in fetal tissues and its proportions are decreased with advancing age (15). Type III collagen synthesis is predominant in injured tissues, during repair and regeneration (16), presumably because regenerating tissues in the early stages resemble fetal development. Our studies suggest that O_2-induced fibrotic lesions may arise from similar injury repair mechanisms. These observations suggest that the initial phases of the fibrotic response of lungs to environmental oxidants may be entirely endogenous to the lungs themselves and not involve systemic influences. Our studies also suggest that lung organ cultures in atmospheres resembling the ambient environment may be a useful model for studying biochemical processes in the lung.

ACKNOWLEDGEMENTS:

These studies were supported by National Institutes of Health Grants HL-19668 and DE-4752. We wish to thank Mrs. Valerie Willwerth for assistance in the preparation of this manuscript.

REFERENCES:

1. Hance, A. J. and Crystal, R. G. (1976) The Biochemical Basis of Pulmonary Function, pp 215-257. Marcel Dekker, New York.

2. Hussain, M. Z., Belton, J. C. and Bhatnagar, R. S. (1978) In Vitro (in press).

3. Hussain, M. Z., Belton, J. C. and Bhatnagar, R. S. (1977) Conference Proceedings of the 4th Joint Conference on Sensing of Environmental Pollutants, pp 532-536. American Chemical Society, Washington, D.C.

4. Woessner, J. F.,Jr. (1961) Arch. Biochem. Biophys. 93,440-447.

5. Juva, K. and Prockop, D. J. (1966) Anal. Biochem. 15, 77-83.

6. Shatkin, A. J. (1969) Fundamental Techniques in Virology, pp 238-241. Academic Press, New York.

7. Rosen, H. (1957) Arch. Biochem. Biophys. 67, 10-15.

8. Bradley, K. H., McConnell, S. D. and Crystal, R. G. (1974) J. Biol. Chem. 249, 2674-2683.

9. Seyer, J. M., Hutcheson, E. T. and Kang, A. H. (1976) J. Clin. Invest. 57, 1498-1507.

10. Sykes, B. C. (1976) FEBS Letters. 61, 180-185.

11. Bowden, D. H. and Adamson, I. Y. R. (1971) Arch. Pathol. 92, 279-283.

12. Massaro, G. D. and Massaro, D. (1974) J. Clin. Invest. 53, 705-709.

13. Chvapil, M. and Peng, Y. M. (1975) Arch. Environ. Health. 30, 528-532.

14. Adamson, I. Y. R. and Bowden, D. H. (1976) Arch. Pathol. Lab. Med. 100, 640-643.

15. Epstein, E. H., Jr. (1974) J. Biol. Chem. 249, 3225-3231.

16. Barnes, M. J. and Morton, L. F. (1976) Biochem. J. 157, 263-266.