Effect of Long-Term Hypoxia on Protein Synthesis in Granuloma and in Some Organs in Rats (35106)

M. Chvapil,[1] J. Hurych, and E. Mirejovská
(Introduced by W. Van Winkle, Jr.)
Department of Experimental Biology, Institute of Industrial Hygiene and Occupational Diseases, Prague, Czechoslovakia

It has been shown that fibrogenic cells are more resistant to low oxygen tension, do not develop degenerative changes (1), and multiply at a higher rate than epithelial cells (2). A logical consequence of these findings seems to be the hypothesis that the chronic exposure of animals to systemic low oxygen tension produces hypoxia in certain tissues with consequential increase in the proportion of fibrogenic cells. Therefore, an increase in collagen biosynthesis would be expected. The biosynthesis of noncollagenous proteins, formed mainly by cells that deteriorate at low oxygen tension, should be decreased.

The aim of this study was to test the hypothesis in artifically induced granuloma and in various tissues of rats exposed to hypoxia for a relatively long period of time.

Material and Methods. Material. Two sets of experiments were performed. In the first experiment, 2-month-old female rats (4 experimental, 4 controls) were exposed for 11 weeks to hypoxia, simulating an altitude corresponding to 7000 m. The exposures were carried out in an air-conditioned low pressure chamber every second day for 24 hr at 24° (3). The first two exposures corresponded to an altitude of 5000 m. The control rats were kept in the same chamber, but at normal atmospheric conditions, to eliminate the possible effect of environment or manipulation. The experimental rats were exposed 31 times; the controls, 21 times. Eleven days before the last exposure, two polyurethane sponges, in the form of a prism (7 × 7 × 30 mm) weighing 75 mg each, were implanted into the dorsal region of the rat. ^{14}C-Proline (U), specific activity 45.0 mCi/mmole, in a dose of 12.8 μCi/100 g of body weight, was administered ip 24 hr before killing. The average body weight of the control rats at the time of killing was 270 g, and 215 g for rats exposed to hypoxia.

In the second experiment, the same arrangement of exposures was applied but the rats were 1 month older, body weight of controls (3 animals) was 305 g, and 265 g for the experimental rats (3 animals). The sponges (2/rat) were removed the seventh day after implantation. ^{14}C-Proline (U) was administered in a dose of 12.4 μCi/100 g body weight 24 hr before sacrificing the rats.

Methods. The granuloma tissues, as well as those organs included in this study, were carefully cleaned from surrounding tissues, frozen in liquid oxygen, and pulverized in a mortar.

In the first experiment, the homogenate of the granuloma was extracted twice for 24 hr with 12 ml of 0.45 *M* NaCl, pH 7.4. The extracts were combined and precipitated with 50% trichloroacetic acid to a final concentration of 15% at 4°. After centrifugation, the soluble collagen was extracted from the sediment with hot trichloroacetic acid (3 times for 20 min at 90°; 5 ml of 0.3 *M* acid/1 g of tissue) (4). The insoluble collagen was extracted from the insoluble pellet in 0.45 *M* NaCl using the same method. From the other organs the total collagen was extracted by the method of Fitch *et al.* (4). Trichloroacetic acid and low molecular components from the combined extracts were removed by dialysis against tap water. The samples were evaporated and then hydrolyzed (16 hr at

[1] Present address: Division of Surgical Biology, Department of Surgery, University of Arizona School of Medicine, Tucson, Arizona.

TABLE I. Effect of Long-Term Hypoxia on the Synthesis of Collagenous and Noncollagenous Proteins in Granuloma Tissue.

Protein fraction	Expt. 1 (11 days)		Expt. 2 (7 days)	
	Control	Hypoxia	Control	Hypoxia
Hydroxyproline in collagen extractable into 0.45 *M* NaCl				
μmole/granuloma	0.35	0.37	0.49	0.94
cpm/μmole	2570	5820	975	1205
Hydroxyproline in collagen insoluble in 0.45 *M* NaCl				
μmoles/granuloma	11.6	14.1	14.4	18.7
cpm/μmole	1264	1028	829	681
Proline in non-collagenous proteins				
μmoles/granuloma	15.6	17.2	9.0	10.6
cpm/μmole	3225	2350	1505	976
Polymerization index				
$\frac{\text{Hyp in collagen soluble in 0.45 } M \text{ NaCl (cpm/granuloma)}}{\text{Hyp in collagen insoluble in 0.45 } M \text{ NaCl (cpm/granuloma)}} \times 100$	6.1	14.9	4.0	8.9

105°, 6 *N* HCl). The pellet left after the extraction with hot trichloroacetic acid was washed with cold trichloracetic acid, ethanol, ethanol:ether (1:4) and ether and represented noncollagenous proteins. Hydrolysis was carried out as mentioned above. Hydroxyproline was determined according to Stegemann (5) and proline according to Troll and Lindsley (6). The separation and isolation of hydroxyproline and proline for radioactive measurement was carried out on a Dowex 50-X8 column (1 × 30 cm). For elution, 1.5 *N* HCl was used and 10-ml fractions were collected. The radioactive proline from noncollagenous proteins was isolated according to Peterkofsky and Prockop (7). For the radioactivity assays a Packard Tri-Carb liquid scintillation spectrometer, Model 3365, was used. The efficiency for collagen samples (water solutions) ranged from 59–61%; for noncollagenous protein samples (toluene solutions), from 75–77%.

In the second experiment the same technique was used for the isolation of different protein fractions. To isolate the low molecular weight hydroxyproline fraction, the 0.45 *M* NaCl extract was dialyzed against 40 ml of distilled water three times. The dialysate containing free- and peptide-bound hydroxyproline was evaporated and desalted on a Dowex 50-X4 column. After the hydrolysis, hydroxyproline was isolated as described above. The nondialyzable residue was centrifuged and soluble collagen was extracted from the sediment into hot trichloracetic acid.

Data presented in Table IV were treated statistically for analysis of variance. Significant differences within 95% confidence limit are italicized.

Results. Hypoxia and protein synthesis in the granuloma tissue. The granuloma formed around subcutaneously implanted sponges shows the most active synthesis of collagenous hydroxyproline among all tissues studied. When the same type of collagenous protein is compared in various tissues (see Tables I and IV (data for insoluble or total collagen), the specific activity is highest in the granuloma tissue. This difference is even more pronounced as the data on specific activity of total collagen from the liver, heart, lung, and small intestine include soluble collagens with relatively high radioactivity. The synthesis of newly-formed collagen, represented by the 0.45 *M* NaCl extract, was significantly stimulated by hypoxia in both independent experiments as is shown by the increase of the specific activity of collagenous ^{14}C-hydroxyproline (Table I).

No changes in the total radioactivity of insoluble collagen were found in either exper-

iment; however, in both experiments the amount of insoluble collagen measured by its hydroxyproline content was increased. In the second experiment (7-day-old granuloma), the amount of neutral salt-soluble collagen was also higher in animals exposed to hypoxia than in the controls. It appears, therefore, that the long-term exposure to hypoxia stimulates collagen synthesis in granuloma tissue. On the other hand, under the same experimental conditions there was an inhibition of noncollagenous protein formation in both experiments, as shown in Table I.

Effect of hypoxia on collagen polymerization. As shown elsewhere (8), the stabilization of the collagen molecule is related to the concentration of oxygen. The ratio of extractable to residual collagen proteins in granuloma tissue of control and hypoxia-treated rats was measured. As shown in Table I, there is more of the soluble forms of collagen in granulomas of rats subjected to hypoxia. In younger granuloma tissue (7 days) the specific activity of soluble collagen is less than that in 11-day-old granuloma, but at both sampling periods the polymerization is inhibited by the exposure to hypoxia.

Effect of hypoxia on collagen degradation. It has been commonly agreed that low molecular weight compounds containing hydroxyproline are degradation products of newly-formed collagenous proteins (9). Furthermore, it is known that abnormal oxygen tension affects the lysososmal membranes and releases the enzymes that may participate in the collagen breakdown (10). Therefore, the amount and the radioactivity of those components in granuloma tissue that are of low molecular weight and contain hydroxyproline were studied. No effect from long-term hypoxia was observed (see Table II). The considerably higher specific activities of dialyzable ^{14}C hydroxyproline compared with 0.45 *M* NaCl soluble collagenous ^{14}C-hydroxyproline have been constantly found. However, no relation between the pool of soluble collagen and the pool of dialyzable hydroxyproline was found.

Effect of hypoxia on the extent of collagenous proline hydroxylation in granuloma tissue. In a recent study on embryonal skin slices it was shown that at low oxygen concentrations only a partial hydroxylation of collagenous proline occurs (8). In this study, the possibility was investigated that even during long-term hypoxia leading, presumably, to tissue hypoxia (at least in granuloma) a hydroxyproline-deficient collagen could be formed. For the evaluation of the degree of hydroxylation we used the ratio of the specific activity of hydroxyproline to proline. The results (Table III) obtained with highly purified collagen showed the same specific activity of both collagenous proline and hydroxyproline, isolated from either the control rats or from the rats adapted to hypoxia. The hydroxylation of collagenous proline was, therefore, not affected by hypoxia.

TABLE II. Effect of Hypoxia on the Amount and Radioactivity of Dialyzable Forms of Hydroxyproline in Granuloma Tissue.[a]

Sample	Dialyzable hydroxyproline (μmole/granuloma)	Dialyzable hydroxyproline (cpm/μmole)
Control	0.097	4120
	0.117	5300
	0.105	5600
Hypoxia	0.137	4500
	0.124	4600
	0.081	4600

[a] Data refer to the second experiment. Each sample represents granuloma tissue pooled from two different sponges of the same rat.

Effect of hypoxia on protein synthesis in some other organs. The reaction pattern of protein synthesis in individual organs of ani-

TABLE III. Effect of Hypoxia on the Hydroxylation of Collagenous Proline in Granuloma Tissue.[a]

Sample	Pro/Hyp	(cpm/μmole) ^{14}C Hyp	(cpm/μmole) ^{14}C Pro	^{14}C Hyp/^{14}C Pro
Control	1.12	1006	1040	0.97
	1.11	1522	1280	1.19
Hypoxia	1.26	884	740	1.19
	1.14	1172	1040	1.13

[a] The data refer to purified insoluble collagen (1st expt.). The ratio Pro/Hyp is used as criterion of purity of isolated protein, the ratio ^{14}C Hyp/^{14}C Pro refers to the degree of hydroxylation.

TABLE IV. Effect of Long-Term Hypoxia on Protein Synthesis in Various Tissues.[a]

Tissue	Expt.	Collagen (as ^{14}C hydroxyproline) (cpm/μmole) Control	Hypoxia	Collagen (as ^{14}C hydroxyproline) (cpm/organ) Control	Hypoxia	Noncollagenous proteins (as ^{14}C proline) (cpm/μmole) Control	Hypoxia	Noncollagenous proteins (as ^{14}C proline) (cpm/organ $\times 10^{-3}$) Control	Hypoxia
Liver	1	793	739	7195	6180	2533	2177	*921*	*780*
	2	645	650	9579	8813	1478	1246	*1058*	*756*
Heart	1	*409*	*192*	*1270*	*965*	*1896*	*1185*	*47*	*31*
Lung	1	182	141	2830	2730	*3062*	*1709*	*101*	*65*
	2	198	165	3695	3325	1497	1170	*96*	*74*
Small intestine	1	*173*	*94*	*1310*[b]	*920*[b]	*3777*	*2423*	*57*[b]	*36*[b]

[a] Summary of the results of both experiments. Every value is the average of three (2nd expt.) or four (1st expt.) independent analyses. Couples of data italicized are significantly different at 95% confidence limit. In all tissues total collagen was extracted into hot 0.3 *M* trichloroacetic acid. In spite of exposure to hypoxia the wet weights of individual organs did not change and amounted for liver: 8.58 $\pm$ 1.34 (controls) and 7.80 $\pm$ 1.27 (hypoxia); heart: 0.76 $\pm$ 0.11 (controls) and 0.78 $\pm$ 0.14 (hypoxia), and for the lung: 1.25 $\pm$ 0.27 (controls) and 1.43 $\pm$ 0.21 (hypoxia).

[b] Expressed as cpm/g of wet tissue.

mals exposed to chronic hypoxia differs considerably. The most common observation is a decrease in noncollagenous protein synthesis in rat liver, heart, small intestine, and lung (Table IV). No significant changes were found in the synthesis of collagen in the liver and the lung. In the heart and small intestine, the long-term hypoxia inhibited the incorporation of radioactivity into collagenous hydroxyproline (Table IV).

Discussion. A dissociation in the biosynthetic pattern for two types of proteins in the same tissue after exposure to long-term hypoxia is demonstrated for the first time. The decrease in noncollagenous protein synthesis was uniformly demonstrated in the granuloma tissue as well as in the four other organs studied. This result agrees with the findings of many other investigators on the inhibiting effect of hypoxia on the synthesis of globular proteins and/or on the activity of most enzymes (11–13). On the other hand, these data show that in the artifically stimulated granuloma tissue, hypoxia led to an increased collagen synthesis in rats adapted to simulated altitude hypoxia. It is not surprising that such stimulation has been found in this structure if it is realized that during certain periods of granuloma development due to impairment of microcirculation a local tissue hypoxia develops (14, 15) which is further exaggerated by exposure to systemic hypoxia. As no effect of hypoxia on the extent of collagen hydroxylation has been found, we believe that the pO_2 in the granuloma tissue was not low enough to limit the function of protocollagen proline hydroxylase (16).

The observation of the stimulating effect of hypoxia on collagen synthesis and of the inhibitory effect on noncollagenous protein formation agrees with the finding of the difference in susceptibility of various cells to oxygen. The exposure to low oxygen exaggerates the local hypoxia in granuloma tissue, and this enhances mitosis in fibrogenic cells, thus increasing the proportion of fibroblasts in the granuloma. Among other cells present in 7- and 11-day-old granuloma, epithelial cells show a functional deterioration; this may explain the decrease in production of noncollagenous proteins. It must be noted that the body weight growth of rats exposed to hypoxia was approximately 20% less than that of the controls. This might explain the decrease of the noncollagenous protein synthesis but does not explain the stimulation of collagen formation.

Our results on stimulation of collagen synthesis in granuloma tissue by long-term hypoxia seem to be in conflict with papers

showing decreased wound healing at low oxygen tensions (17, 18). A partial explanation may lie in the finding that hypoxia inhibits polymerization of collagen. In most investigations the measure of healing was the gain in tensile strength; this is not proportional to the amount of collagen present, but to the degree of maturation of collagen fibrils. Another important difference is that in the present experiments, rats were used which were adapted to hypoxia first and subsequently the sponges were implanted.

It is difficult to interpret the findings on collagen synthesis in organs such as the liver, lung, heart, or small intestine, as each organ has its own characteristic course of adaptation mechanisms to long-term exposure to hypoxia. For instance, hypoxia is a factor known to produce cardiomegaly with an increase in collagenous stroma (19). This is a dynamic process involving the different rates of the growth of muscle and collagen and ending in cardiofibrosis. Nevertheless, in this study no change in heart weight and collagen content was found, but the radioactivity of collagenous hydroxyproline decreased significantly. This may be due to the experimental method employed wherein the synthesis of proteins during a 24-hr period only was measured. The previous adaptive changes in the individual tissues were disregarded. However, it can be concluded that there occurs in certain organs a divergence of the biosynthetic pattern for collagenous and noncollagenous protein after long-term hypoxia.

Summary. In rats adapted first to hypoxia simulating 7000-m altitude, further exposures to low oxygen tensions stimulated collagen synthesis only in the granuloma tissue and had no effect on the degradation of collagen. The polymerization of soluble forms into less soluble collagen was decreased. In the liver and lung, collagen synthesis did not change. In the heart and small intestine, collagen synthesis was significantly decreased. The formation of noncollagenous proteins was significantly inhibited in all tissues studied. No effect of hypoxia on hydroxylations of collagenous proline was observed.

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Received March 30, 1970. P.S.E.B.M., 1970, Vol. 135.