Increase of collagen synthesis in pulmonary arteries of monocrotaline-treated rats

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Summary. Collagen synthesis in the pulmonary artery in animals with pulmonary hypertension was studied using monocrotaline-treated rats as a model. Incorporation of ^{14}C-labeled proline into collagenase-digestible protein in the pulmonary artery was found to be 4–5 times higher in monocrotaline-treated rats than in control rats.

Monocrotaline is a toxic pyrrolizidine alkaloid isolated from the legume, *Crotalaria spectabilis.* Administration of this substance, either by long-term feeding[1,2] or single s.c. injection[3], results in pulmonary hypertension in association with interstitial pneumonitis and right ventricular hypertrophy. It has been shown further that pulmonary hypertension can be produced in puppies by a single s.c. injection of monocrotaline[4].

Recently Ooshima et al.[5,6] and Iwatsuki et al.[7] have demonstrated that collagen synthesis, as assessed by 3 biochemical markers, prolyl hydroxylase activity, in vitro incorporation of ^{14}C-labeled proline into collagenase-digestible protein and collagen content, is increased in the aorta, mesenteric arteries and brain arteries of spontaneously hypertensive rats as well as DOCA-induced hypertensive rats. Iwatsuki et al.[7] suggest that the stimulus for vascular collagen synthesis is provided by a direct effect of the increased pressure on the arterial wall.

The present experiment was carried out to investigate whether collagen synthesis was increased in the pulmonary artery when blood pressure was elevated using monocrotaline-treated rats as the model of pulmonary hypertension.

Materials and methods. 5-week-old male Sprague-Dawley rats were used. They were housed in individual cages with free access to a commercial diet (CE-2, CLEA Japan Inc., Tokyo) and water. Monocrotaline solution was prepared as follows: 200 mg of crystalline monocrotaline (S.B. Penick Co., New York) was dissolved in 1.2 ml of 1 N HCl, diluted with distilled water to about 5 ml and neutralized with 0.5 N NaOH; the volume was then adjusted to 10 ml with distilled water.

30 rats were given a single s.c. injection of monocrotaline in a dose of 40 mg/kg, and as the control, 50 rats received a s.c. injection of 0.1 ml of saline. All rats were killed under Nembutal anesthesia either 21 days or 22 days after injection, and the trunk of the pulmonary artery, and the aortic arch, together with the descending thoracic aorta and the heart, were excised. The heart was fixed in 10% formalin for 48 h, and both ventricles and septum were weighed separately for evaluation of right ventricular hypertrophy.

Collagen synthesis in the pulmonary artery and aorta was assessed by in vitro incorporation of ^{14}C-labeled proline into collagenase-digestible protein according to the methods of Ooshima et al.[5] and Peterkofsky et al.[8]: 100–150 mg of tissue samples were pooled, minced and incubated in 1.25 ml of Earle's balanced salts solution buffered with 28 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], containing 10 µCi of ^{14}C-labeled proline (New England Nuclear, Boston) for 5 h at 37 °C under 95% O_2 and 5% CO_2. After that, the samples were chilled in ice water, and the tissues were homogenized in 1–3 ml of 0.05 M Tris-HCl (pH 7.4). The homogenates were then dialyzed for 48 h against 4 changes of the same buffer, and trichloroacetic acid (TCA) was added to give a final concentration of 5%. The resultant precipitates were collected by centrifugation, washed thrice with 5% TCA, twice with ethanol-ether (3:1, v/v), once with ether, and dried. The dried materials were dissolved in 0.1 N NaOH at a concentration of 2.5–5.0 mg/ml, and used as the substrate solution for collagenase-digestion. The collagenase-digestion was carried out by incubating the substrate solution with purified collagenase in the following reaction mixture at 37 °C for 3 h with shaking: 0.2 ml of the substrate solution, 60 µmoles of N-ethylmaleimide (Wako Junyaku Co., Osaka), 0.25 µmoles of $CaCl_2$, 2 mg of collagen (Sigma Co., St. Louis), 50 µg of purified collagenase (Seikagaku Kogyo Co., Tokyo), and 0.05 M Tris-HCl (pH 7.4) containing 0.005 M $CaCl_2$ to adjust the final volume to 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 10% TCA containing 0.5% tannic acid. After centrifugation, the supernatants were transferred to vials with 10 ml of Triton-Omuniflour (2:1, v/v, New England Nuclear, Boston) and counted in a liquid scintillation counter, Model L-SC-652, Aloka Co. Ltd, Tokyo.

Results and discussion. In the control group, all rats survived throughout the experimental period, while in monocrotaline-treated group, 2 of 30 rats died from pulmonary edema within 20 days after injection. The body weight and heart weight are shown in table 1. Right ventricular hypertrophy as assessed by weight of right ventricle and septum appeared in monocrotaline-treated rats. This finding indicates the occurrence of pulmonary hypertension in the rats, since correlative physiological and morphological studies by Kay et al.[9] and Katayama et al.[10] showed that administration of monocrotaline to rats increased pulmonary arterial blood pressure in association with right ventricular hypertrophy.

Table 2 shows the collagen synthesis in the pulmonary artery and aorta, as indicated by incorporation of ^{14}C-labeled proline into collagenase-digestible protein. In control rats, this marker of collagen synthesis was found to be much lower in the pulmonary artery (499 ± 149 cpm/mg protein) than in the aorta (3485 ± 1680 cpm/mg protein), while in monocrotaline-treated rats, these 2 arteries showed similar levels of collagen synthesis; 2283 ± 420 cpm/mg protein in the pulmonary artery and 2464 ± 650 cpm/mg protein in the aorta. The increase of collagen synthesis in

Table 1. Body and heart weights of rats following a single injection of monocrotaline

Group	Body weight (g)		Heart weight (relative weight[a])		
	Initial	Final	Total	Left ventricle	Right ventricle and spetum
Test	135.7 ± 8.2	255.4 ± 27.9[b]	510 ± 63[b]	125 ± 18[b]	301 ± 44[b]
Control	136.1 ± 4.7	304.8 ± 14.8	354 ± 7	107 ± 16	200 ± 19

[a] $p < 0.01$; [b] relative weight expressed in percentage of final body weight × 10^3.

the pulmonary artery of monocrotaline-treated rats is apparently related to the elevation of pulmonary arterial blood pressure as indicated by the right ventricular hypertrophy (table 1). According to Kay et al.[9] and Katayama et al.[10] the systolic pressure of the pulmonary artery ranges from 20 to 30 mm Hg in control rats, and after monocrotaline treatment, it can reach 100 mm Hg or more.

Ooshima et al.[5,6] and Iwatsuki et al.[7] have reported that in 2 models of hypertension in rat, there is increased synthesis of collagen in various arteries of the systemic circulation including the aorta, mesenteric arteries and brain arteries, and that such effects are reversed when blood pressure is lowered by antihypertensive drugs. On the basis of these findings, they have postulated that elevation of blood pressure itself initiates the changes in collagen synthesis in the arterial vessels. The present results suggest that this possibility is also applicable in the case of the pulmonary circulation.

Table 2. In vitro incorporation of ^{14}C-labeled proline into collagenase-digestible protein of arterial tissues isolated from monocrotaline-treated rats and control rats

Group	Pulmonary artery	Aorta
Test	2,283 ± 420[a] (6)[b]	2,464 ± 650 (6)[b]
Control	499 ± 149 (5)[b]	3,485 ± 1,680 (9)[b]

[a] $p < 0.01$; [b] the numerals in the parentheses indicate the number of pools of tissue, each comprising 4–10 rats. Rats were killed 21 or 22 days after an injection of 40 mg/kg of monocrotaline. The pulmonary artery and aorta were excised, minced and pooled. Samples of tissue were incubated with ^{14}C-labeled proline, homogenized, dialyzed, and treated with collagenase. Values for incorporation of ^{14}C-labeled proline are expressed as cpm/mg protein. The values represent the mean ± SD.

1 J.J. Lalich, Path. Microbiol. *27*, 965 (1964).
2 J.H. Turner and J.J. Lalich, Archs Path. *79*, 409 (1965).
3 Y. Hayashi, J.F. Hussa and J.J. Lalich, Lab. Invest. *16*, 875 (1967).
4 B.F. Atkinson, A.S. Katz, D. Knight, G.G. Pietra and A.P. Fishman, Lab. Invest. *36*, 354 (1977).
5 A. Ooshima, G.C. Fuller, G.J. Cardinale, S. Spector and S. Udenfriend, Proc. nat. Acad. Sci USA *71*, 3019 (1974).
6 A. Ooshima, G.C. Fuller, G.J. Cardinale, S. Spector and S. Udenfriend, Science *190*, 898 (1975).
7 K. Iwatsuki, G.J. Cardinale, S. Spector and S. Udenfriend, Science *198*, 403 (1977).
8 V. Peterkofsky and R. Diegelman, Biochemistry *10*, 988 (1971).
9 J.M. Kay, P. Harris and D. Heath, Thorax *22*, 176 (1967).
10 H. Katayama and Y. Hayashi, Trans. Soc. Path. Japan *60*, 160 (1970).

Antidepressant drugs elevate rat pineal and plasma melatonin*

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Summary. Acute administration in the mid-light phase of a number of antidepressant drugs of different pharmacological profiles elevated pineal and plasma melatonin (measured by radioimmunoassay). Following chronic treatment with the tricyclic antidepressant clomipramine, the elevation was significantly reduced. This may be an effect of reduced ß-adrenergic receptor sensitivity after chronic clomipramine administration, analogous to other findings of reduced ß-adrenergic receptor binding and reduced noradrenaline-sensitive adenylate-cyclase response.

The development of radioimmunoassay[2] and gas-chromatography-mass-spectography methods[3] for measuring the pineal hormone melatonin has permitted physiological studies of circulating levels in man. Plasma melatonin undergoes circadian variations that reflect variations in pineal β-adrenergic receptor sensitivity. This has led to growing interest in the measurement of melatonin in affective disorders, since both desynchronization of circadian rhythms[4] and β-adrenergic sensitivity changes[5] have been postulated to occur in depression and mania. Preliminary clinical investigations indicate a lowering of melatonin in depression and an increase of it in mania[6,7] that would be in accordance with the latter hypothesis. The antidepressant drug desmethylimipramine can increase the conversion of tryptophan to melatonin[8], as well as increasing the activity of the enzyme N-acetyl-tranferase in vitro and in vivo[9]. We therefore treated animals with a number of different antidepressant drugs (and potential antidepressants) and measured melatonin in the pineal gland and plasma after both acute and chronic administration.

Male albino Wistar rats were kept on an light: dark cycle of 12:12 (lights on at 05.00 h) for 3 weeks prior to use, at a temperature of 25 °C, with food and water ad libitum. In acute drug experiments, the animals were injected i.p. in the middle of the light phase and decapitated 2 h later; in chronic experiments, the animals were injected i.p. daily 15 min before lights off and decapitated 2 h after a final injection. Trunk blood was collected in heparin and centrifuged, and the plasma frozen; pineals were rapidly removed and frozen.

Melatonin concentrations were determined by a previously described radioimmunoassay[10]. Extensive validation using the classical techniques of parallelism of diluted aliquots of extract, cross-reactivity measurements, and chromatographic identity of immunoreactivity with standard melatonin, showed that the antibody used in these studies (K244) shows good specificity and sufficient sensitivity for reproducibility[11]. However, radioimmunoassay specificity is always open to question since every sample cannot be validated. We found in some of the pineal extracts from animals treated with antidepressant drugs that TLC, (in $CHCH_3$:methanol 9:1 using tritiated melatonin as marker) revealed a fast-running immunoreactive spot. The R_f did not correspond to any indoles similar in structure to melatonin that have been tested for cross-reactivity against K244. TLC of plasma from the same animals did not show the fast-running spot found in the pineals. However, TLC of plasma from animals treated with Ro 11-2465 showed about 30% of the immunoreactivity to be slow-running and not associated with melatonin. Thus, certain groups might have pineal melatonin values slightly increased due to this unknown component; this caveat applies only to animals