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Monocrotaline-Induced Pulmonary Endothelial Dysfunction in Rats (41847)

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Abstract. To study the role of endothelial damage in the pathogenesis of lung injury induced by the pyrrolizidine alkaloid monocrotaline, three functions (angiotensin converting enzyme (ACE) activity, plasminogen activator (PLA) activity, and prostacyclin (PGI_2) production) associated with the pulmonary endothelium were examined, and were correlated with pulmonary arterial perfusion and ultrastructure in rats receiving monocrotaline in their drinking water (20 mg/liter) for 1–12 weeks. Lung ACE activity increased after 1 week of monocrotaline, then decreased steadily from 1 to 6 weeks, before plateauing at approximately 55% of normal. PLA activity in monocrotaline-treated lungs did not change significantly for the first 2 weeks, then decreased to 59 and 79% of the control value after 6 and 12 weeks, respectively. In contrast, PGI_2 production increased progressively, reaching 140 and 270% of the control level after 6 and 12 weeks of monocrotaline treatment, respectively. These endothelial functional changes were not accompanied by significant changes in pulmonary arterial perfusion as visualized by ^{99m}Tc lung scans. Electron microscopy of monocrotaline-treated lungs revealed endothelial damage (perivascular and subendothelial edema, degeneration) starting at 1 week, and inflammatory and hemorrhagic reactions starting at 2 weeks. At 6 and 12 weeks, monocrotaline-treated rats also exhibited increased pulmonary arterial wall thickness, right heart enlargement, and cardio- and hepatomegaly. Thus, monocrotaline-induced pulmonary injury is accompanied, and in some cases preceded, by structural and functional abnormalities in the pulmonary endothelium.

The pyrrolizidine alkaloid monocrotaline induces pulmonary hypertension and hepatotoxicity in rats (1, 2). Consumption of the parent plant, *Crotalaria spectabilis,* is a veterinary problem in grazing animals, and may represent a potential hazard for herbal tea users (3). While the mechanism of monocrotaline toxicity is not clear, it is accompanied by well-documented histopathologic and ultrastructural changes in the pulmonary endothelium (4–9). In contrast, relatively little is known concerning endothelial *function* in monocrotaline-treated lungs. Monocrotaline has been reported to decrease both angiotensin converting enzyme activity (10–12) and 5-hydroxytryptamine clearance (13, 14) in rat lung, although single injections of large doses were employed in three of these studies (10, 11, 13). Oral administration of monocrotaline may require several weeks to produce pulmonary endothelial dysfunction (12, 14).

Angiotensin-I-converting enzyme (ACE) activity, plasminogen activator (PLA) activity, and prostacyclin (PGI_2) production are among the many metabolic activities of the pulmonary endothelium, and are known individually to respond to a variety of insults (15–18). As a group, these three metabolic activities have been found in this laboratory to be a convenient and fairly sensitive index of endothelial function in irradiated rat lung (19). Therefore in order to study the role of endothelial dysfunction in the pathogenesis of monocrotaline-induced lung injury, we measured pulmonary ACE and PLA activities and PGI_2 production, and correlated these with arterial perfusion and ultrastructure in rats receiving monocrotaline in their drinking water for up to 12 weeks.

Materials and Methods. *Experimental design.* Male Sprague–Dawley rats (Harlan Industries, Madison, Wisc.) weighing 325–350 g were housed in pairs at 23 ± 1°C and given standard lab chow (Ralston Purina Co., St. Louis, Mo.) *ad libitum.* Half of the animals were provided with tap water, and half were given water containing monocrotaline (S. B. Penick Co., Lyndhurst, N.J.) at a concentra-

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tion of 20 mg/liter. Periodic measurements revealed relatively constant daily water consumption of 35 ml/rat, resulting in a drug dose of approximately 2.0 mg/kg/day at the start of the experiment. The monocrotaline concentration in the drinking water was not increased as the animals gained weight. Body weight was recorded weekly.

Lung perfusion scans. Pulmonary arterial perfusion scans were obtained from randomly selected animals in both treatment groups after 1, 2, 4, 6, or 12 weeks. Animals were anesthetized with intraperitoneal sodium pentobarbital (Diabutal, Diamond Industries, Des Moines, Iowa), 35 mg/kg, and were injected with approximately 300 μCi of ^{99m}Tc-labeled macroaggregated albumin in 0.3 ml of 0.9% NaCl in the femoral vein. Thoracic scintigrams were obtained from an Ohio Nuclear gamma camera with a pinhole collimater, as described previously (20).

Autopsy procedure. Groups of six to eight rats were autopsied after 1–12 weeks of treatment. Animals were anesthetized with pentobarbital, and a blood sample was obtained from the abdominal aorta. The blood was allowed to clot at room temperature for 30 min, and the serum was separated and stored at −20°C. The abdominal aorta was then transected to exsanguinate the animal. The left lung was cut into quarters, perpendicular to the spinal axis, and termed the cephalic, subcephalic, supracaudal, and caudal regions, respectively, from the cephalic end.

ACE activity. The subcephalic region of the left lung was weighed, frozen in liquid N_2 and stored at −20°C. ACE activity in homogenized lung and blood serum was determined within 72 hr of autopsy by the spectrophotometric method of Cushman and Cheung (21), using the synthetic substrate hippuryl-L-histidyl-L-leucine (Sigma Chemical Co., St. Louis, Mo.). Protein concentration in the lung homogenate and serum was determined by the biuret method. ACE activity was expressed in mU/mg protein, as described previously (22).

PLA activity. The cephalic and caudal regions of the left lung were frozen in liquid N_2 and stored at −20°C. PLA activity was determined by the fibrin plate lysis method of Astrup and Albrechtsen (23). The data were expressed in area (mm^2) of the fibrin plate lysed under standard *in vitro* conditions as described previously (24).

PGI_2 production. The supracaudal region of the left lung was minced, weighed, and incubated in 3.0 ml of Dulbecco's phosphate-buffered saline (Gibco, Grand Island, N.Y.) containing glucose (1 mg/ml) for 10 min at 37°C. Aliquots of the incubation medium were transferred to a tube containing aspirin (final concentration 1 m*M*) to prevent further production of PGI_2. The samples were frozen and stored at −70°C until radioimmunoassayed for 6-keto-$PGF_{1\alpha}$ the stable metabolite of PGI_2 (New England Nuclear, Boston, Mass.). The data were expressed as nanograms 6-keto-$PGF_{1\alpha}$ produced per milligram wet weight of lung during the 10-min incubation.

Electron microscopy. Two control and two monocrotaline-treated rats were sacrificed for electron microscopy at each treatment time. The animals were anesthetized with pentobarbital and exsanguinated. The thoracic organs were removed *en bloc,* and the lungs were perfused via the trachea with 4°C glutaraldehyde–paraformaldehyde fixative at a pressure of 22 cm of water, as described previously (25). Lung samples were postfixed in osmium tetroxide and embedded in epoxy resin. Thick sections were stained with toluidine blue, and areas of interest were thin-sectioned, stained with uranyl acetate and lead citrate, and examined on a Hitachi HU-12 transmission electron microscope.

Organ weights. The liver was weighed at autopsy, and the heart was weighed after fixation in 10% phosphate-buffered Formalin (pH = 7.4). Organ weight was expressed as a fraction of body weight. The heart was dissected, and the right ventricle (RV) and the left ventricle plus septum (LV + S) were weighed separately. Right ventricular hypertrophy was evaluated on the basis of RV/LV + S weight ratios.

Statistical analysis. The effect of time and monocrotaline administration on ACE and PLA activity and PGI_2 production was determined by two-way analysis of variance (26).

Results. *Pulmonary arterial perfusion.* Thoracic scintigrams indicated that neither control nor monocrotaline-treated rats developed pulmonary arterial perfusion defects at any time throughout the study.

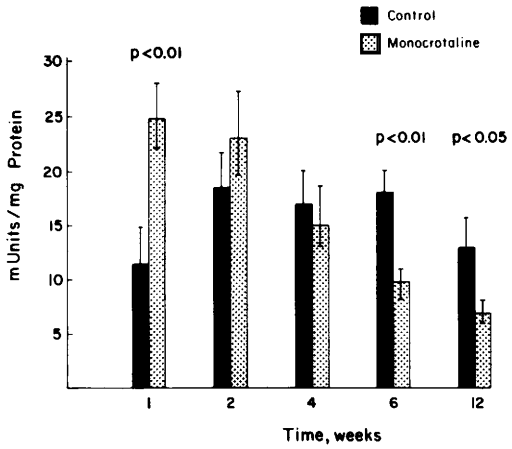

FIG. 1. Pulmonary angiotensin-I-converting enzyme activity (A-I-CE) (mU/mg protein) as a function of treatment time in control rats (solid bars) and animals receiving monocrotaline, 2.0 mg/kg body wt/day, po (stippled bars). Mean ± SEM; six to eight animals per group.

ACE activity. ACE activity in the lung of control animals ranged from 11.2 ± 3.8 to 18.8 ± 6.1 mU/mg protein, and did not change significantly with time (Fig. 1). In contrast, monocrotaline-treated rats exhibited a significant ($P < 0.01$) increase in ACE activity after 1 week, followed by a steady decrease in enzyme activity to approximately 55% of the corresponding control values at 6 and 12 weeks (Fig. 1). Changes in lung ACE activity were not accompanied by significant changes in serum activity of the enzyme (Table I), nor by significant changes in lung wet weight (unpublished data).

PLA activity. PLA activity in the lung of control animals increased steadily from a value of 141 ± 9 mm^2 of fibrin plate lysed to 193 ± 5 mm^2 lysed during the 12-week study (Fig. 2). In rats receiving monocrotaline, PLA activity was normal for the first 2 weeks, then decreased to 86, 59, and 79% of the corresponding control activity at 4, 6, and 12 weeks, respectively (Fig. 2).

PGI_2 production. Monocrotaline had no significant effect on the production of 6-keto-$PGF_{1\alpha}$ by the lung of rats sacrificed after 1, 2, or 4 weeks of treatment (Fig. 3). After 6 and 12 weeks, however, production of this prostaglandin increased to 140 and 270% of the corresponding control levels, respectively (Fig. 3).

Ultrastructural changes. Electron microscopic evaluation of monocrotaline-treated lungs revealed capillary subendothelial edema, and venous and arterial perivascular edema starting at 1 week. Interstitial inflammatory

TABLE I. THE EFFECT OF MONOCROTALINE ON BODY AND ORGAN WEIGHTS AND SERUM ACE ACTIVITY IN RATS

Endpoint	Group[a]	Weeks of treatment				
		1	2	4	6	12
Body weight (g)	CON	353 ± 6[b]	379 ± 5	410 ± 10	443 ± 19	488 ± 9
	MONO	359 ± 16	387 ± 7	378 ± 7*	391 ± 8*	447 ± 14
Heart weight (mg/100 g body wt)	CON	340 ± 10	320 ± 10	331 ± 7	330 ± 21	310 ± 10
	MONO	320 ± 10	330 ± 10	325 ± 11	390 ± 20*	360 ± 20*
RV/LV + S[c]	CON	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
	MONO	0.10 ± 0.01	0.09 ± 0.02	0.13 ± 0.01	0.16 ± 0.01*	0.15 ± 0.02*
Liver weight (g/100 g body wt)	CON	3.66 ± 0.10	4.06 ± 0.27	3.74 ± 0.11	3.83 ± 0.07	3.59 ± 0.12
	MONO	3.79 ± 0.13	3.83 ± 0.08	3.84 ± 0.09	4.11 ± 0.14*	3.92 ± 0.15*
Serum ACE (mU/mg protein)	CON	0.69 ± 0.05	0.68 ± 0.04	0.64 ± 0.07	0.80 ± 0.10	0.67 ± 0.10
	MONO	0.68 ± 0.07	0.59 ± 0.05	0.66 ± 0.05	0.51 ± 0.07	0.70 ± 0.11

[a] Control group (CON) or monocrotaline-treated, 2.0 mg/kg body wt/day, po (MONO).
[b] Mean ± SEM; six to eight animals/group.
[c] Right ventricle (RV) weight as a fraction of the weight of the left ventricle plus septum (LV + S) of the heart.
* Different from CON, $P < 0.05$.

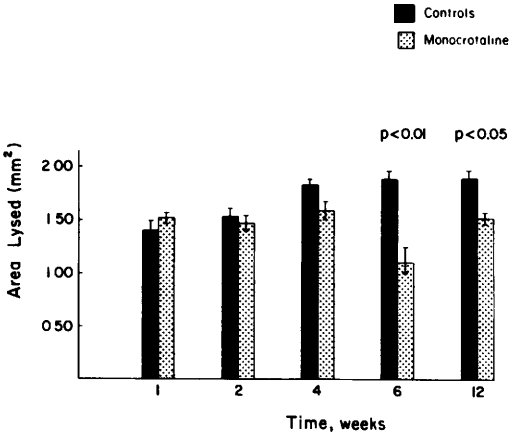

FIG. 2. Pulmonary plasminogen activator activity (mm^2 of fibrin plate lysed) as a function of treatment time in control rats (solid bars) and animals receiving monocrotaline, 2.0 mg/kg body wt/day, po (stippled bars). Mean ± SEM; six to eight animals per group.

cell infiltrates around small veins and arteries were observed at 2 weeks, as were interstitial red blood cells. While the edema tended to subside by 6 weeks, interstitial inflammatory cells and red blood cells accumulated through 12 weeks of treatment. Increased interstitial collagen was observed in the monocrotaline-treated lungs by six weeks. Capillary endothelial fragmentation and degeneration also were evident by 6 weeks (Fig. 4).

Starting at 4 weeks, there was a progressive increase in the thickness of the walls of pulmonary arteries and arterioles in the monocrotaline-treated animals. This increased wall thickness was the result of a combination of smooth muscle cell hypertrophy and hyperplasia, increased connective tissue and edema in the media, and multiple elastic laminae.

Body and organ weight. Animals treated with monocrotaline for 4 weeks or longer developed a slight but significant retardation of body weight gain (Table I). Monocrotaline-treated animals also exhibited significant cardio- and hepatomegaly at 6 and 12 weeks. The cardiomegaly was associated specifically with a progressive right ventricular hypertrophy from the 4th to the 12th weeks of monocrotaline administration (Table I).

Discussion. These data demonstrate that monocrotaline-induced lung injury is accompanied by structural and functional abnormalities in the pulmonary endothelium. Endothelial dysfunction in the lungs of rats receiving oral monocrotaline includes decreased ACE and PLA activity, and increased PGI_2 production observed in the present study, as well as decreased serotonin uptake reported previously (14). Pulmonary dysfunction in our monocrotaline-treated rats (2.0 mg/kg/day, po) requires 4–6 weeks to become significant, which may explain why Huxtable *et al.* (14) failed to detect changes in lung activity of ACE, 5'-nucleotidase, and monoamine oxidase in rats consuming monocrotaline (3.6 mg/kg/day, po) for 3 weeks. Large single injections of monocrotaline (60–105 mg/kg, sc), in contrast, suppress ACE activity and serotonin uptake within 10 days (10, 11, 13).

After 4–6 weeks of oral monocrotaline, rats also develop changes associated with pulmonary hypertension and cor pulmonale, i.e., increased pulmonary arterial wall thickness, right ventricular hypertrophy, cardiomegaly, and hepatomegaly. The development of these anatomic manifestations of monocrotaline induced pulmonary hypertension is preceded by a transient increase in lung ACE activity, and by ultrastructural evidence of endothelial injury (capillary subendothelial edema and

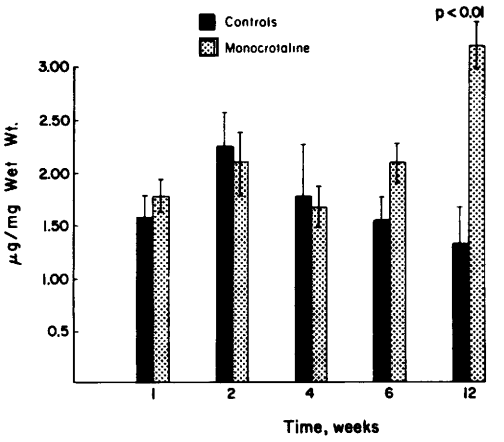

FIG. 3. Pulmonary prostacyclin (PGI_2) production (ng 6-keto-$PGF_{1\alpha}$/mg wet weight) during a 10-min *in vitro* incubation as a function of treatment time in control rats (solid bars) and animals receiving monocrotaline, 2.0 mg/kg body wt/day, po (stippled bars). Mean ± SEM; six to eight animals per group.

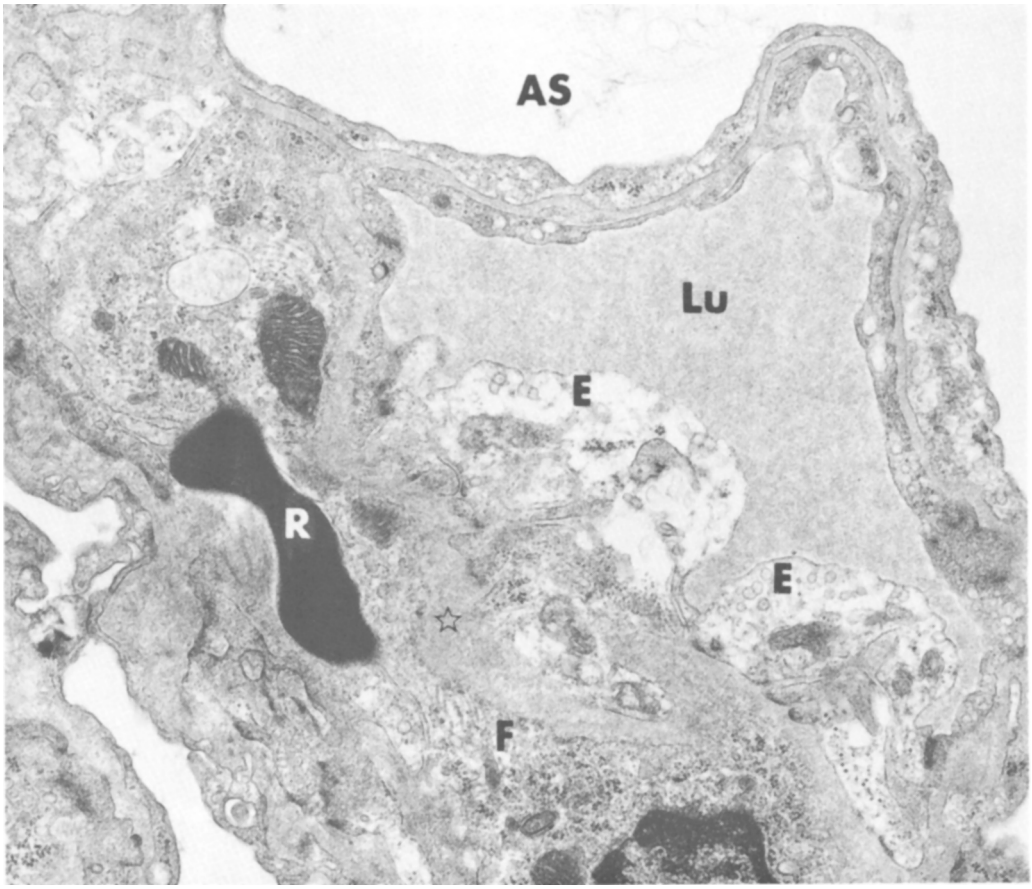

FIG. 4. Electron micrograph of the lung of a 6-week monocrotaline-treated rat. Two endothelial cells (E) of the capillary are electron lucent and apparently degenerated. The subendothelial space of these cells is swollen (*). A red blood cell (R) is present in the interstitium. Fibroblasts (F) contain numerous ribosomes and polysomes. As, airspace; Lu, lumen of capillary. ×14,740.

perivascular edema of small arteries and veins) after 1 week of drug treatment. After single injections of large doses of monocrotaline, however, lung ACE activity falls steadily, without exhibiting an initial increase in enzyme activity (10). Both decreased ACE activity, presumably resulting in reduced angiotensin II-mediated vasoconstriction, and increased PGI_2 production, presumably resulting in increased vasodilation, would appear to be appropriate compensatory responses to pulmonary hypertension in monocrotaline-treated lungs. That inappropriate ACE activity may play a role in the pathogenesis of experimental pulmonary hypertension is suggested by the observation that ACE inhibitors ameliorate the hypertensive response to both monocrotaline (12) and chronic hypoxia (27, 28). Despite significant changes in ACE activity in the lungs of monocrotaline-treated rats, blood serum levels of the enzyme do not fluctuate. Thus serum ACE activity is not a reliable index of lung status in this model. Kay *et al.* (10) found a similar disparity between lung and serum ACE activity following monocrotaline injections.

The monocrotaline-induced endothelial dysfunction observed in the present study is not accompanied by pulmonary arterial perfusion defects as determined on ^{99m}Tc lung scans of anesthetized animals. In contrast, Meyrick *et al.* (9) found that rats fed *Crotalaria*

seeds exhibited narrowing of arterial lumens and reduced background haze in arteriograms performed on thawed frozen lungs injected intraarterially with Micropaque and gelatin (60°C) at a pressure of 100 cm H_2O. While the two techniques differ in many respects, together they may indicate normal regional distribution of perfusion through a partially occluded, overly muscularized (9, 10) arterial network in monocrotaline-treated lungs. This then might lead to increased pulmonary arterial pressure (14), and the development of right heart enlargement.

The changes in ACE and PLA activity and in PGI_2 production observed in the present study are not unique to monocrotaline-induced pulmonary injury. Identical responses occur in the lung following ionizing radiation (19, 22, 24, 25) and bleomycin (Ward *et al.*, unpublished data). This endothelial dysfunction implies perturbations in both the hemodynamic and fibrinolytic activity of the injured lung. While the time of onset of endothelial dysfunction differs among the several insults (and among routes of administration of the same insult), the pattern of response is qualitatively similar in the several models of lung injury. These metabolic abnormalities may reflect endothelial cell death, or dysfunction of surviving cells, or a combination of the two. Electron micrographs reveal endothelial degeneration and fragmentation coincident with the development of abnormal metabolic activity.

In conclusion, the present data indicate that endothelial injury is a prominent feature of the lung's response to the pyrrolizidine alkaloid monocrotaline. Monocrotaline-induced pulmonary damage is accompanied by, and in some cases preceded by both structural and functional abnormalities in the pulmonary endothelium.

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