Effect of monocrotaline ingestion on the distribution of protein and angiotensin converting enzyme activity in the rat lung

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ABSTRACT The alveolar accumulation of protein and angiotensin converting enzyme activity was compared with the development of right ventricular hypertrophy in male rats after different periods of monocrotaline exposure. Total doses of monocrotaline were varied by dividing the animals into three groups in which ingestion was limited to three, seven, and 15 days. These groups were studied 21 days after the start of monocrotaline exposure and compared with a group treated continuously for 28 days. The total lung weight increased after three or more days of treatment, while after seven days there was significant alveolar accumulation of protein, which was paralleled by an increase in angiotensin converting enzyme activity in alveolar lavage fluid. Identical changes also occurred after 15 and 28 days of exposure to monocrotaline. Lung angiotensin converting enzyme activity was decreased after three days' ingestion of monocrotaline and did not alter further with longer periods of exposure. None of these effects of monocrotaline in the three and seven day treatment groups was associated with right ventricular hypertrophy, which occurred only in animals treated for 15 or more days. The effects of monocrotaline ingestion on the lung were dose related and had no causal relationship to the development of right ventricular hypertrophy.

Exposure to monocrotaline produces acute endothelial injury and the later development of pulmonary hypertension and cor pulmonale in the rat and has been used as a model for studying the pathophysiology of pulmonary vascular disease occurring in man.¹⁻³ A single subcutaneous dose of monocrotaline (40-105 mg/kg) will cause alveolar wall interstitial oedema and structural changes in endothelial cells within 24 hours, and a microvascular protein leak that is detectable three days after administration.^{4,5} A causal link has been proposed between this protein leak, which is maximal at seven days, and the development of right ventricular hypertrophy, which usually occurs 10-14 days after parenteral treatment.^{5,6} The same cardiovascular changes can, however, be induced with cumulative but smaller total doses of monocrotaline administered by the oral route.⁷⁻⁹ These regimens have produced a sequence of pulmonary circulatory changes that lead to pulmonary hypertension and right ventricular hyper-

trophy,^{8,9} but more slowly than after single dose parenteral treatment.^{6,9}

Monocrotaline treatment produces various changes in the metabolic functions of the pulmonary circulation.^{6,8,10} In single dose studies a reduction in lung angiotensin converting enzyme activity (ACE, EC 3.4.15.1) has been reported to follow the development of pulmonary hypertension, and this may represent a regulatory mechanism to control blood volume and the increase in pulmonary artery pressure.^{2,6,11} No change in lung ACE activity, however, occurred in rats on low dose oral monocrotaline regimens causing the same pulmonary circulatory changes.^{8,10,12} Thus the change in lung ACE activity might be related to the development of pulmonary hypertension and right ventricular hypertrophy, but might equally be an unrelated effect due to the use of high dose monocrotaline regimens.

We undertook the present study to define the effects of various oral doses of monocrotaline on the evolution of protein accumulation in the alveolus and the distribution of ACE activity in serum, lung, and alveolar lavage fluid, and on the relationship of these to the development of right ventricular hypertrophy.

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Methods

EXPERIMENTAL PROTOCOL

Twenty five Sprague-Dawley strain male rats (Medical Research Council), with an initial mean body weight of 90 (SD 14.2) g, received either monocrotaline (Warner Lambert Ltd, USA) in their drinking water (20 mg/l 62 μ mol/l) or tap water ad libitum. Groups of six or seven animals ingested monocrotaline for three, seven, or 15 days. The effect of treatment was studied at 21 days and compared with a control group receiving water for 21 days. Another group was studied after 28 days of continuous monocrotaline ingestion to provide a high dose control group. All samples were obtained under pentobarbitone anaesthesia (120 mg/kg intraperitoneally) (May and Baker Ltd). Blood collected from the inferior vena cava was allowed to clot on ice. The pulmonary circulation was freed of blood by the infusion of 10 ml of ice cold bicarbonate buffered normal saline (pH 7.4). The lungs were then lavaged via a tracheal cannula with 5 ml of ice cold buffered saline, which for 30 seconds was repeatedly removed and reinjected. Lungs were removed, blotted dry, and stored with all other specimens at -20°C until analysis. Hearts were fixed in buffered formol saline (pH 7.6) and dissected after four days' fixation. The right ventricular free wall was dissected from the left ventricle at its junction with the septum. The daily ingestion of monocrotaline was monitored by weighing the drinking water bottles of rats kept in groups of four.

ASSAY PROCEDURES

A weighed portion of lung (about 100 mg) was homogenised in 2 ml of phosphate buffered isotonic saline (pH 8.3) in a hand held homogeniser. After centrifugation (3000 rev/min for 30 min, 4°C) the supernatant was collected and the total protein content and ACE activity were measured. Protein was determined by a colorimetric method (Bio Rad Ltd, UK) with bovine serum albumin as the standard.¹³ A standard curve was prepared for each assay run. The

average interassay variation was 12% (4–15% as coefficient of variation) over the assay standard curve for 12 assays. Angiotensin converting enzyme activity was determined spectrophotometrically¹⁴ with hippuryl-L-histidyl-L-leucine as the substrate (CBC Ltd, UK). For two control sera in 18 assays, the inter-assay variation was 5.9% and 8.6% (as coefficient of variation). Dry weight was determined by drying at 60°C until a constant weight was achieved for a pre-weighed portion of lung.

Statistical methods

The significance of differences between groups was assessed with an unpaired Student's *t* test after an F test for the equality of variance. A value of *p* less than 0.05 was taken to indicate statistical significance. Correlation coefficients and regression lines were calculated from standard formulae.¹⁵

Results

MONOCROTALINE INGESTION

The average intake of monocrotaline solution was 21.1 (2.4) ml/rat/day, giving an average daily ingestion of 424 μg /rat/day.

ANATOMICAL DATA

The body weight was significantly reduced only after 28 days of monocrotaline treatment (table 1). The wet weight of the lung increased after three days of treatment, with a significant increase in lung to body weight ratio and parallel increases in total lung protein, dry weight, and water content that were significant after seven days' monocrotaline exposure (table 2), although individual components did not change significantly as a proportion of total lung mass for any exposure period (table 2). Increase in lung mass was separated from the development of right ventricular hypertrophy, which occurred only after 15 days of monocrotaline treatment (tables 1 and 2).

Table 1 Mean (SD) body and heart weights of rats receiving oral monocrotaline

	Days of monocrotaline treatment*					
	C21	3	7	15	28	C28
Final body weight (g)	226	216	212	206	156†	314
(SD)	(14)	(27)	(20)	(25)	(42)	(9)
RV/LV + S	0.31	0.33	0.34	0.43†	0.65†	0.28
(SD)	(0.05)	(0.05)	(0.05)	(0.09)	(0.09)	(0.02)
No/group	6	6	6	7	4	5

*C21 and C28 are the 21 and 28 day control groups. Animals were studied at 21 days unless stated otherwise.

†*p* < 0.05 in comparison with controls.

RV/LV + S—ratio of weight of free wall of right ventricle to weight of left ventricle and septum.

BIOCHEMICAL DATA

The absolute concentration and lavage to serum (L:S) ratios for protein increased significantly after seven days' monocrotaline treatment (table 3). This was not increased further after 15 days' exposure, although it was after 28 days. The absolute and L:S ACE activity ratio also increased with length of exposure to monocrotaline (table 3). There was a significant correlation between total L:S protein and ACE activity ratios ($r = 0.96$; $p < 0.001$, $df\ 32$). Expression of lavage ACE activity as specific activity of lavage total protein showed a significant reduction after seven and 15

days' monocrotaline ingestion (table 3). Serum ACE activity was significantly raised only after 28 days' monocrotaline ingestion, while serum protein concentration was unaffected (table 3). Total lung protein (mg/lung) was significantly increased after seven days' exposure to monocrotaline and was related to changes in other components of the lung (table 2). Hence there was no significant increase in protein per unit of lung. Total lung ACE activity was significantly decreased by three or more days' monocrotaline treatment (table 4) and was inversely correlated with lung dry weight ($r = 0.74$; $p < 0.001$, $df\ 32$).

Table 2 Mean (SD) weight of lungs and lung components in rats receiving oral monocrotaline

	Days of monocrotaline treatment*					
	C21	3	7	15	28	C28
Lung:body weight	2.48 (0.23)	3.09† (0.17)	3.85† (0.49)	3.71† (0.38)	6.56† (2.7)	2.16 (0.2)
Lung weight (mg)	557 (47)	664 (101)	814† (111)	766† (99)	940† (89)	675 (64)
Dry lung weight (mg)	82 (6)	87 (14)	115† (19)	110† (16)	115† (15)	90 (5)
Dry:total weight (%)	14.7 (1.2)	13.2 (1.2)	14.1 (0.6)	14.4 (1.1)	12.2 (0.8)	14.1 (2.0)
Lung water (mg/lung)	475 (45)	576 (90)	698† (92)	655† (85)	825† (61)	581 (60)
Water:total weight (%)	85.3 (1.0)	86.8 (1.1)	85.9 (0.5)	86.6 (1.0)	87.8 (0.8)	85.3 (1.7)
Lung protein (mg)	27.9 (3.0)	30.6 (6.1)	38.2† (5.8)	33.4† (4.6)	36.3† (7.1)	26.6 (1.8)
Protein:total weight (%)	5.1 (0.7)	4.6 (0.4)	4.7 (0.4)	4.4 (0.5)	3.9 (0.7)	4.1 (0.4)

*C21 and C28 are the 21 and 28 day control groups.

† $p < 0.05$ in comparison with controls.

Table 3 Mean (SD) total protein content and angiotensin converting enzyme (ACE) activity of lavage fluid in rats receiving oral monocrotaline

	Days of monocrotaline treatment*					
	C21	3	7	15	28	C28
Serum ACE (nmol/min/ml)	102.9 (14.6)	114.6 (17.5)	112.9 (25.0)	107.3 (27.9)	129.7† (4.5)	113.5 (6.4)
Serum protein (mg/ml)	43.0 (1.7)	42.3 (4.0)	44.7 (1.5)	44.5 (2.5)	48.3 (7.4)	50.8 (2.9)
Specific activity serum ACE (nmol/min/mg protein)	2.4 (0.33)	2.75 (0.70)	2.51 (0.51)	2.41 (0.60)	2.98† (0.26)	2.24 (0.16)
Lavage ACE (nmol/min/ml)	0.55 (0.22)	0.44 (0.15)	1.17† (0.38)	1.11† (0.50)	2.17† (0.26)	0.50 (0.16)
Lavage protein (mg/ml)	0.22 (0.15)	0.20 (0.08)	0.78† (0.34)	0.75† (0.30)	1.44† (0.24)	0.29 (0.06)
Specific activity of lavage ACE (nmol/min/mg protein)	2.47 (0.61)	2.36 (0.59)	1.59† (0.36)	1.53† (0.57)	1.49 (0.10)	1.72 (0.44)
Lavage:serum ACE ($\times 10^4$)	53.8 (18.1)	38.7 (12.4)	106.9† (30.9)	102.8† (35.1)	165† (30.6)	43.8 (8.9)
Lavage:serum protein ($\times 10^4$)	53.2 (32.0)	48.0 (20.0)	176.3† (69.8)	162.7† (60.2)	317† (26.0)	59.0 (10.0)

Results represent mean and 1 SD for absolute values and for lavage to serum ratios.

*C21 and C28 are the 21 and 28 day control groups respectively.

† $p < 0.05$ in comparison with respective controls.

Table 4 Mean (SD) lung angiotensin converting enzyme (ACE) activity in rats receiving oral monocrotaline

	Days of monocrotaline treatment*					
	C21	3	7	15	28	C28
Total lung ACE activity nmol/min/lung	3852 (282)	3111 (743)	2941† (149)	2948† (463)	2700 (126)	3663 (766)
Lung ACE activity nmol/min/mg protein	139 (10.6)	103† (16.2)	79.2† (12.4)	90† (16.1)	75.6† (13.4)	136.4 (28.7)

*C21 and C28 are the 21 and 28 day control groups respectively.

†p < 0.05 in comparison with controls.

Discussion

Variation of monocrotaline dosage by length of exposure allowed exploration of the temporal relationships of increased lung mass, alveolar protein accumulation, and ACE activity changes to the development of right ventricular hypertrophy. Our rats had an estimated average intake of 424 µg monocrotaline/day (equivalent to 2.01 mg/kg/day) on the basis of the ingestion studies, so that a total dose of 2.97 mg (equivalent to 14.1 mg/kg) was found to induce a significant increase in lung mass and alveolar protein accumulation and a reduction of lung ACE activity without the subsequent development of right ventricular hypertrophy. The same daily dose for 15 days (total 6.4 mg, equivalent to 30.3 mg/kg), however, produced the same changes, but with the addition of right ventricular enlargement. This confirms the advantages of the low dose oral regimen, which allows the demonstration of dose related effects on the pulmonary circulation.^{8,9}

The accumulation of protein in the alveoli of rats treated with monocrotaline for seven or more days was presumed to be related to the presence of a microvascular protein leak.^{4,5} Sugita *et al*⁵ induced a protein leak with a single parenteral dose (40 mg/kg) of monocrotaline. The leak was present after three days, preceded the development of right ventricular hypertrophy by 11 days, and was assumed to have a causal association. An increased permeability to protein was present throughout the 21 days of their study and the authors considered it possible that it was maintained by the raised pulmonary artery pressure. In the present study, however, protein accumulation in the alveolus was a sustained dose dependent effect of monocrotaline and was independent of the development of right ventricular hypertrophy, suggesting that the leak was unlikely to have been maintained by developing pulmonary hypertension. Sugita *et al*⁵ reported no effect on the alveoli of the leak they demonstrated, because albumin concentration was not raised one and three days after the start of monocrotaline ingestion. Inspection of their data, however, shows small but statistically significant increases in alveolar lavage albumin concentrations at one, seven, 14, and 21 days. Their data therefore are not at vari-

ance with our findings of protein accumulation in the alveoli after monocrotaline treatment, although they could not dissociate this from the later development of pulmonary hypertension and right ventricular hypertrophy.

We found differential changes in ACE activity between the circulatory, interstitial, and alveolar compartments of the lung, whereas previously only serum and lung activities have been reported. ACE is likely to have leaked passively into the alveolar space. The parallel change in protein concentration and ACE activity in lavage fluid and the correlation of L:S ratios for ACE and protein supports this. Similar changes in alveolar protein and ACE follow acute injury to the pulmonary endothelium of the rabbit by hyperoxia,¹⁶ but not after acute bacterial endotoxin injury in the mouse.¹⁷ Serum ACE activity may rise after acute endothelial injury as a result of release of enzyme into the circulation, with a corresponding fall in lung enzyme activity.^{18,19} This mechanism was not, however, active in this study as monocrotaline treatment did not cause a rise in serum ACE activity, except after 28 days' treatment, and the reduction of total lung ACE activity was maintained after monocrotaline treatment had ceased. Possibly the fall in ACE specific activity after seven or 15 days' treatment was due to addition of protein synthesised locally²⁰ in response to monocrotaline induced alveolar injury.^{6,21}

The reduction of lung ACE activity after only three days of exposure to monocrotaline has not been reported previously. It implies an early direct effect of monocrotaline on the lung independent of the development of pulmonary hypertension.^{6,9} The sustained reduction of enzyme activity in the lung may be due to several mechanisms. The most obvious explanation is that endothelial ACE content is unchanged and the reduction of ACE activity is apparent rather than real, because of inflammation and hyperplasia in the lung. The inverse correlation between lung dry weight and total or specific activity for lung ACE supports this. Other mechanisms, however, such as a reduction of the pulmonary circulatory surface area secondary to distal pulmonary vascular pruning, may occur after longer treatment periods.⁹ The reduced ability of the pulmonary vascular bed of the rat to generate

angiotensin II after monocrotaline treatment¹¹ also supports this interpretation, but is probably a longer term effect. Kay *et al*⁶ reported reduction of ACE activity 10 days after a single 60 mg/kg dose of monocrotaline, which coincided with a rise in pulmonary vascular resistance and preceded right ventricular hypertrophy. They suggested that this change represented a regulatory mechanism limiting the development of pulmonary hypertension.^{2 6 11} Our findings do not support this hypothesis as the ACE activity of the lung was consistently decreased at monocrotaline doses incapable of inducing right ventricular hypertrophy and preceding pulmonary hypertension.

Reduced lung ACE activity after monocrotaline ingestion confirms the findings of studies using single subcutaneous doses.^{2 6 10} This is at variance with the findings of other workers, who used the same strain of rats and a similar dosage schedule for monocrotaline.^{8 10} They used a microsomal extract of lung to determine ACE activity, and also argued that lung ACE could not be expressed in terms of protein as this changed in a non-uniform way with lung growth.^{10 12} Inspection of their data shows that lung protein, water, and total weight increased in a proportionate manner.¹⁰ Hence the lung protein was 0.148 and 0.146 mg/mg of lung for their control and monocrotaline groups, which is in keeping with our findings.

In summary, small cumulative doses of monocrotaline, incapable of inducing right ventricular hypertrophy, caused a sustained accumulation of protein in the alveolar spaces, increased lung mass, and reduced lung ACE activity. These effects were considered to be due to a dose dependent action of monocrotaline on the pulmonary circulation and the lung, and indicate that alveolar protein accumulation and changes in ACE activity are independent of the development of pulmonary hypertension under the circumstances of the experiments reported here.

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