Serum Copper Concentration as an Index of Cardiopulmonary Injury in Monocrotaline-Treated Rats*

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ABSTRACT

The pyrrolizidine alkaloid monocrotaline produces pulmonary inflammation, hemorrhage, fibrosis, and hypertension. In rats, monocrotaline pneumotoxicity can be ameliorated by cotreatment with inhibitors of angiotensin converting enzyme (ACE), such as CL242817. In the present study, serum and urine copper (Cu) concentrations were evaluated as indices of cardiopulmonary injury in rats sacrificed after six weeks of continuous administration of monocrotaline (0 to 3.6 mg per kg per day, in the drinking water) or CL242817 (60 mg per kg per day, in the feed), or both. Monocrotaline-treated rats exhibited dose-dependent increases in (1) pulmonary histopathology, (2) pulmonary endothelial dysfunction (decreased lung plasminogen activator activity, and increased prostacyclin and thromboxane production), (3) pulmonary hydroxyproline (collagen) content, and (4) cardiac right ventricular hypertrophy (an anatomic correlate of pulmonary hypertension). The severity of cardiopulmonary damage was accompanied by a dose-dependent elevation in serum Cu concentration. Serum iron concentration, in contrast, did not change. Urinary Cu concentration correlated roughly with that of serum, but the variability within groups was high. Cotreatment with the ACE inhibitor CL242817 not only ameliorated monocrotaline-induced right heart enlargement and lung hydroxyproline accumulation but also reduced the hypercupremia in monocrotaline-treated rats. Thus, serum copper concentration appears to be an accurate and minimally invasive index of monocrotaline pneumotoxicity in this model of pulmonary hypertension.

Introduction

Hypercupremia accompanies several lung diseases in humans, including cigarette smoking,7 emphysema,32 lung cancer,15 and primary pulmonary hypertension.2 Experimental pneumotoxins such as carrageenan,5 legionellosis,13 thoracic irradiation,33 and monocrotaline12 also produce an elevation in serum
copper.

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copper concentration in rodents. It has been suggested that this relationship between hypercupremia and pneumotoxicity may be more than coincidental, that excess copper in the serum may contribute to the pathogenesis of lung injury.\textsuperscript{2,12,33} Supporting this hypothesis are the observations that inhaled or injected copper salts produce lung damage,\textsuperscript{1,9,10,18} and that copper is toxic to endothelial cells\textsuperscript{29} and pulmonary macrophages\textsuperscript{27} \textit{in vitro}.

On the other hand, copper also is regarded as protective under some conditions of lung injury, particularly oxidant stresses. The element is cofactor of one form of superoxide dismutase, and copper-deficient animals are sensitive to both hyperoxic lung damage\textsuperscript{17} and emphysema.\textsuperscript{28} Thus, the role of copper in the development of lung injury appears to be equivocal.\textsuperscript{34} Existing data do not allow one to distinguish whether the hypercupremia which accompanies spontaneous or induced lung damage is a coincidence, an epiphenomenon, a nonspecific acute phase reactant, a specific index, or a cause of lung injury.

In the present study, the authors sought to examine more closely the relationship between serum copper concentration and cardiopulmonary damage in the monocrotaline model of pneumotoxicity. Monocrotaline is a pyrrolizidine alkaloid extracted from the shrub \textit{Croton spectabilis}. The alkaloid is both hepatotoxic and pneumotoxic, producing pulmonary edema, inflammation, hemorrhage and fibrosis.\textsuperscript{19} While the cellular mechanism of monocrotaline pneumotoxicity is not known, the pulmonary endothelium appears to be an early and sensitive target.\textsuperscript{16,19,20,21,23} Monocrotaline-induced pulmonary vascular reactions include endothelial dysfunction,\textsuperscript{20,21,23} increased permeability,\textsuperscript{31} and muscularization and mural thickening of the small arteries and arterioles. These reactions lead to pulmonary hypertension, right heart enlargement, and cor pulmonale.\textsuperscript{19} Ingestion of the parent plant is both a veterinary problem and a health hazard in herbal tea users.

Administration of the alkaloid has been proposed as an animal model of pulmonary hypertension and adult respiratory distress syndrome.\textsuperscript{6,14,22} Monocrotaline-induced cardiopulmonary injury can be ameliorated by cotreatment with a variety of agents,\textsuperscript{6,14} including inhibitors of angiotensin converting enzyme (ACE).\textsuperscript{24,25} Monocrotaline administration is an appropriate model for the study of the relationship between hypercupremia and pneumotoxicity, since Ganey and Roth have reported recently that serum copper concentration increases significantly within eight days after a single s.c. injection of monocrotaline pyrrole in rats.\textsuperscript{12} If the relationship between hypercupremia and monocrotaline-induced cardiopulmonary damage is more than coincidental, then dose-dependent increases in monocrotaline pneumotoxicity should be accompanied by dose-dependent increases in serum copper concentration. Furthermore, ACE inhibitors such as CL242817 should ameliorate not only monocrotaline cardiopulmonary damage but also the alkaloid-induced hypercupremia. In the present study, these two hypotheses were tested in rats consuming monocrotaline (0 to 3.6 mg per kg per day) or CL242817 (60 mg per kg per day), or both, continuously for six weeks. Urinary copper concentration also was evaluated as an index of cardiopulmonary status.

\textbf{Methods}

\textbf{MONOCROTALINE DOSE-RESPONSE EXPERIMENT}

Male Sprague-Dawley rats* weighing 200 ± 20 g were housed in pairs at 23 ±

* Charles River, Boston, MA.
1°C and consumed standard lab chow† ad libitum. Animals were randomly assigned to one of three treatment groups whose drinking water contained 0, 20, or 30 mg of monocrotaline‡ per liter. Water consumption was measured weekly, and remained constant at 32 to 35 ml per rat per day, resulting in a monocrotaline regimen of 0, 2.4, or 3.6 mg per kg body weight per day, p.o., respectively. On the day before autopsy, individual rats were placed in a metabolism cage, and urine was collected from 08:00 to 12:00 hrs and then stored at −20°C until assayed for copper concentration.

All animals were sacrificed after six weeks of continuous monocrotaline administration. The rats were anesthetized with sodium pentobarbital (35 mg per kg, i.p.), and were exsanguinated by syringe from the abdominal aorta. Blood was allowed to clot at room temperature; serum was collected by centrifugation, then stored at −20°C until assayed for copper and iron concentration.

The thoracic organs were dissected en bloc, and the superior, medial, and inferior lobes of the right lung were ligated at the trachea and removed. The superior lobe of the right lung was weighed, frozen in liquid nitrogen, then stored at −20°C until assayed for hydroxyproline content by the method of Stegemann and Stalder. Data were expressed as μg hydroxyproline per right superior lobe (RSL). The medial lobe of the right lung was frozen in liquid N₂ and stored at −20°C until assayed for plasminogen activator activity by the fibrin plate lysis method. Data were expressed as area of the fibrin plate lysed under standard in vitro conditions. The inferior lobe of the right lung was immediately minced, weighed, and incubated for 10 min at 37°C in 3.0 ml of Dulbecco’s phosphate buffered saline§ containing glucose (one mg per ml). Aliquots of the incubation medium then were mixed with aspirin solution (final concentration: two mM), left at room temperature for one hr, frozen in liquid N₂, and stored at −20°C. Within one month after autopsy, the concentration of prostacyclin (PGI₂) and thromboxane A₂ (TXA₂) in the samples was determined by radioimmunoassay of their stable metabolites, 6-keto-prostaglandin_{1α} (6-keto-PGF₁₀) and thromboxane B₂ (TXB₂), respectively. Samples assayed by these commercially available radioimmunoassay (RIA) kits yield stable results for at least six months when stored at −20°C. Data were expressed as ng of prostanoid produced per mg of minced lung during the 10 min incubation.

The left lung was inflated via the trachea with 10 percent buffered formalin at a pressure of 22 cm of water. A midsagittal section of the left lung was embedded in paraffin, sectioned at four μm, and stained with hematoxylin-eosin. Each lung sample was evaluated under light microscopy by two investigators unfamiliar with the treatment history of the animal. Pulmonary histopathology was scored on a scale from 0 (normal) to 4 (severe).

The formalin-fixed heart was weighed, then dissected into the right ventricle (RV) and the left ventricle plus septum (LV + S). Right heart enlargement was evaluated on the basis of the RV/LV + S weight ratio. Total copper concentration in the serum and urine was measured by atomic absorption spectroscopy at 324.7 nm as described by Dawson et al. Serum total iron was determined using a commercially available ferrozine kit.***

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† Ralston-Purina, St. Louis, MO.
‡ Aldrich Chemical Co., Milwaukee, WI.
§ Grand Island Biological Co., Grand Island, NY.
|| New England Nuclear, Boston, MA
‡ Molteni et al, unpublished data
** Sigma Chemical Co., St. Louis, MO.
TABLE I
Cardiopulmonary Injury in Monocrotaline-Treated Rats*

<table>
<thead>
<tr>
<th>Response</th>
<th>0</th>
<th>2.4</th>
<th>3.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (percent)</td>
<td>100</td>
<td>83</td>
<td>75</td>
</tr>
<tr>
<td>Body weight (gram)</td>
<td>445 ± 5</td>
<td>381 ± 19†</td>
<td>363 ± 9†</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>1368 ± 57</td>
<td>1534 ± 94</td>
<td>1733 ± 50†</td>
</tr>
<tr>
<td>Left ventricle weight (mg/g body weight)</td>
<td>3.08 ± 0.09</td>
<td>4.05 ± 0.27†</td>
<td>4.79 ± 0.26†</td>
</tr>
<tr>
<td>Left ventricle weight (mg/g body weight)</td>
<td>709 ± 42</td>
<td>613 ± 18</td>
<td>580 ± 41</td>
</tr>
<tr>
<td>Right ventricle weight (mg/g body weight)</td>
<td>1.60 ± 0.08</td>
<td>1.61 ± 0.05</td>
<td>1.60 ± 0.14</td>
</tr>
<tr>
<td>Right ventricle weight (mg/g body weight)</td>
<td>177 ± 9</td>
<td>316 ± 31†</td>
<td>369 ± 20†</td>
</tr>
<tr>
<td>RV/LV+S§ (percent)</td>
<td>25.0 ± 1.4</td>
<td>51.6 ± 4.7†</td>
<td>64.0 ± 3.1†</td>
</tr>
<tr>
<td>Lung histopathology (0.0-4.0)</td>
<td>1.1 ± 0.3</td>
<td>2.8 ± 0.2†</td>
<td>2.8 ± 0.3†</td>
</tr>
<tr>
<td>Lung PLA activity (mm² lysed)</td>
<td>85 ± 5</td>
<td>32 ± 19†</td>
<td>0 ± 0†</td>
</tr>
<tr>
<td>Lung TXA₂ production (ng/mg Wet/10 min)</td>
<td>0.28 ± 0.04</td>
<td>0.48 ± 0.02†</td>
<td>0.54 ± 0.06†</td>
</tr>
<tr>
<td>Lung wet weight (mg/BRL)‡</td>
<td>138 ± 10</td>
<td>202 ± 13†</td>
<td>227 ± 17†</td>
</tr>
</tbody>
</table>

*Rats were sacrificed after six weeks of continuous monocrotaline ingestion. Mean ± SEM; n = 6-8.
† Different from control (0 mg), p < 0.05
‡ Different from 2.4 mg monocrotaline, p < 0.05
§ Weight ratio of cardiac right ventricle to the left ventricle plus septum
¶ Wet weight of the superior lobe of the right lung (RSL)

CL242817 Modification Experiment

The source and housing of rats in this experiment were similar to the previously described monocrotaline dose-response study. Animals were randomly assigned to one of four treatment groups:

Group 1: control; drank tap water, and consumed standard powdered chow.

Group 2: CL242817; drank tap water, and consumed powdered chow containing 0.12 percent (w/w) of CL242817.††

Group 3: monocrotaline; drank water containing 20 mg monocrotaline‡‡ per liter, and consumed standard powdered chow.

Group 4: monocrotaline plus CL242817; drank water as in Group 3, and consumed powdered chow as in Group 2.

Food and water consumption were measured weekly and remained constant at 20 to 22 g and 32 to 35 ml per rat per day, respectively, in all groups. This resulted in average daily doses of 2.4 mg per kg for monocrotaline and 60 mg per kg for CL242817.

Animals were sacrificed after six weeks of continuous drug administration. Lung hydroxyproline content, RV/LV+S, and serum copper and iron concentrations were determined as described previously. All data were subjected to analysis of variance, and the significance of differences between group means was determined by the Newman-Keuls test.35

Results

Monocrotaline Dose-Response Experiment (Table I)

Monocrotaline-treated rats exhibited a dose-dependent decrease in both survival and body weight. The alkaloid also produced cardiomegaly and right ventricular hypertrophy, whose severity
increased with increasing monocrotaline dose. Monocrotaline pneumotoxicity presented the well-documented pulmonary interstitial edema, inflammation, hemorrhage and fibrosis, accompanied by a dramatic increase in wall thickness of the pulmonary small arteries and arterioles. When scored on a scale of 0.0 (normal), 1.0 (mild), 2.0 (moderate), 3.0 (marked) and 4.0 (severe), average histopathology scores of 1.1 ± 0.3, 2.8 ± 0.2 and 2.8 ± 0.3 were obtained from the groups receiving 0, 2.4, and 3.6 mg monocrotaline per kg per d, respectively. The light micrographic evidence of monocrotaline-induced pulmonary interstitial fibrosis was accompanied by a dose-dependent increase in lung hydroxyproline content (figure 1). Monocrotaline-treated animals also exhibited pulmonary endothelial dysfunction, as indicated by a dose-dependent decrease in lung plasminogen activator (PLA) activity, and increases in lung prostacyclin (figure 1) and thromboxane production.

These cardiopulmonary responses to monocrotaline were accompanied by a significant (p < 0.05), dose-dependent increase in serum copper concentration, from a value of 115 ± 9 µg per dl in control animals to 177 ± 16 µg per dl in the group receiving 3.6 mg monocrotaline per kg per d (figure 1). Urine copper concentration also increased with increasing monocrotaline dose, but the variability within groups was high, and the differences between group means were not significant (figure 1).

CL242817 Modification Experiment (Table II)

Animals receiving CL242817 alone were indistinguishable from the control group. Monocrotaline-treated rats, in contrast, exhibited a significant increase in both RV/LV+S and lung hydroxyproline content, and these reactions were ameliorated significantly by cotreatment with CL242817. Monocrotaline-treated rats also exhibited a significant hypercupremia, which was reduced in animals receiving concomitant CL242817. Serum iron concentration, in contrast, ranged from 143 ± 6 to 156 ± 8 µg per dl and was independent of treatment.

Discussion

These data demonstrate that elevations in serum copper concentration accompany cardiopulmonary damage in monocrotaline-treated rats. The fact that hypercupremia and pneumotoxicity exhibit similar monocrotaline dose-dependency is consistent with the hypothesis that the former may represent a systemic index of monocrotaline lung injury. Whether serum copper concentration is a specific index of pulmonary injury or is a nonspecific response to inflammatory processes in general remains to be determined. These data further demonstrate that cotreatment with the ACE inhibitor CL242817 not only ameliorates monocrotaline-induced pulmonary fibrosis and right ventricular hypertrophy, but also reduces the hypercupremia observed in rats receiving the alkaloid. This finding is consistent with the hypothesis that elevations in serum copper concentration may in fact contribute to the development of lung damage.

Serum iron concentration, in contrast, is not influenced significantly by either monocrotaline or CL242817 administration. If excess serum copper is a causal factor in monocrotaline-induced cardiopulmonary damage, one would predict that hypercupremia would precede evidence of pneumotoxicity. In the present study that prediction was not tested since serum was collected only at the six-week autopsy time. Nevertheless, Ganey and Roth found that serum copper concentration increased significantly
Figure 1. Lung pros-tacyclin (PGI₂) production (upper left), lung hydroxy-proline (HP) content (upper right), serum copper (Cu) concentration (lower left), and urine copper concentration (lower right) as a function of monocrotaline dose in rats sacrificed after six weeks of continuous drug administration in the drinking water. Mean ± SEM; n = 6 - 8. * = different from control (0 mg), p < 0.05.

within eight days after a single injection of monocrotaline pyrrole, i.e., approximately one week before the onset of significant pulmonary hypertension.

In several respects, the present data from monocrotaline-treated rats resemble those obtained recently by our laboratory in another rat model of lung injury, i.e., thoracic irradiation. In both models there is a dose-dependent relationship between pneumotoxicity and hypercupremia. Furthermore, in both models, cotreatment with a modifying agent reduces both the lung damage and the elevation in serum copper concentration. The modifier used in the radiation study, D-penicillamine, is a known chelator and mobilizer of tissue copper. This action, in fact, may be the basis of its suppressive effect on serum copper concentration in irradiated rats. In contrast, CL242817 does not to our knowledge chelate cations. Thus, its ability to reduce serum copper concen-
**TABLE II**

**Relationship Between Cardiopulmonary Injury and Serum Copper and Iron Concentrations in Monocrotaline-treated Rats: Monification by the ACE Inhibitor CL242817**

<table>
<thead>
<tr>
<th>Response</th>
<th>Control</th>
<th>CL242817 (CL)</th>
<th>Monocrotaline (MONO)</th>
<th>MONO + CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV/LV+S § (percent)</td>
<td>20.1 ± 0.8</td>
<td>25.2 ± 1.1</td>
<td>42.5 ± 2.6†</td>
<td>32.9 ± 2.5</td>
</tr>
<tr>
<td>Lung HP I I (μg/RSL)</td>
<td>300 ± 13</td>
<td>301 ± 28</td>
<td>402 ± 17†</td>
<td>353 ± 22†‡</td>
</tr>
<tr>
<td>Serum copper (μg/dl)</td>
<td>97 ± 3</td>
<td>97 ± 2</td>
<td>132 ± 7</td>
<td>119 ± 4</td>
</tr>
<tr>
<td>Serum iron (μg/dl)</td>
<td>156 ± 8</td>
<td>146 ± 6</td>
<td>144 ± 9</td>
<td>143 ± 6</td>
</tr>
</tbody>
</table>

*Rats were sacrificed after six weeks of continuous ingestion of monocrotaline (2.4 mg/kg/d) or CL242817 (60 mg/kg/day) or both. Mean ± SEM; n = 10.
†Different from control, p < 0.05.
‡Different from monocrotaline, p < 0.05.
§Weight ratio of cardiac right ventricle to the left ventricle plus septum.
Iμg Hydroxyproline per right superior lobe of the lung.

Hypercupremia and pneumotoxicity in monocrotaline-treated rats is less easily explained. It is unknown at the present time whether the increase in total serum copper concentration represents changes in the free or bound fraction of this element, or whether it is related to variations in ceruloplasmin levels. Ceruloplasmin is a copper carrier protein which binds approximately 90 percent of the metal and which is elevated as an acute phase reactant in conditions of inflammation or injury such as thermal burns. No information is yet available about ceruloplasmin levels in monocrotaline-induced injury, and further studies are needed to answer this important question.

In conclusion, hypercupremia and pneumotoxicity respond similarly with respect to monocrotaline dose and CL242817 modification. While this may be only a coincidence, the present data suggest that serum copper concentration is an accurate and minimally invasive index of cardiopulmonary damage in monocrotaline-treated rats. Unfortunately, urinary copper concentration appears to be too variable to serve as a noninvasive index of lung damage in this model.

**Acknowledgments**

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**References**

7. Davidoff, G. N., Votaw, M. L., Coon,


