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PROLONGED ACTIVATION OF RAT LUNG ORNITHINE DECARBOXYLASE IN MONOCROTALINE-INDUCED PULMONARY HYPERTENSION*

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Abstract—Polyamines are believed to have an essential role in cellular growth and differentiation. Activation of ornithine decarboxylase, the initial rate-limiting enzyme in polyamine biosynthesis, is an early event characteristic of cell growth processes. Monocrotaline-induced pneumotoxicity is associated with cellular hypertrophy and proliferation, most notably pronounced medial thickening of pulmonary arterioles and hypertrophy of right ventricular myocardial cells. We reasoned that polyamines may be causally related to these events and, therefore, elevations in lung and right ventricular ornithine decarboxylase activities might precede development of pulmonary hypertension and right ventricular hypertrophy. To test this hypothesis adult male rats were given monocrotaline (105 mg/kg, s.c.); lung and right and left ventricular ornithine decarboxylase activities, pulmonary artery pressure, and right ventricular hypertrophy were assessed at 1, 4, 7, 10, 14, 16 and 21 days post treatment. Lung ornithine decarboxylase activity was increased approximately 8-fold on day 1 and remained elevated through day 7. Right ventricular ornithine decarboxylase activity was not elevated above control values at any time. Elevated pulmonary artery pressure and right ventricular hypertrophy were not apparent until day 16 and day 14 respectively. Thus, sustained activation of lung ornithine decarboxylase occurred at least 1 week prior to the development of pulmonary hypertension, suggesting that polyamines may play an important role in the pulmonary vascular remodeling that accompanies monocrotaline-induced pneumotoxicity.

Administration of monocrotaline (MCT), a pyrrolizidine alkaloid found in plants, to rats has provided a useful model to study development and progression of hypertensive pulmonary vascular disease [1–5]. The evolution of MCT-induced pulmonary hypertension is a complex and apparently multi-stage progressive process which has been proposed [2] to involve the following general chronological sequence: capillary endothelial cell damage, pulmonary vascular hypertrophy and hyperplasia, increased pulmonary vascular resistance and ultimately pulmonary arterial hypertension and right ventricular hypertrophy. The biochemical mechanisms by which MCT causes these toxic effects in the lung are unknown.

The polyamines spermidine and spermine and their diamine precursor, putrescine, are generally believed to have an essential role in cellular proliferation and differentiation [6–11]. Formation of putrescine from ornithine is catalyzed by ornithine decarboxylase (EC 4.1.1.17, L-ornithine decarboxylase, ODC), the initial and rate-limiting enzyme in polyamine biosynthesis [6–11]. Decarboxylation of *S*-adenosyl-L-methionine by *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50, AdoMet-DC) is the first step in the generation of *n*-propylamine groups which are necessary for the formation of spermidine from putrescine and spermine from spermidine [6–11]. Several laboratories have provided evidence that chemically-induced pulmonary cell injury is coupled to increased lung polyamine synthesis and subsequent lung cell proliferation [12, 13]. Other studies have indicated that cardiac hypertrophy induced by several different stimuli is associated with increased cardiac polyamine biosynthesis [7, 14–18].

Since hypertrophy and proliferation of lung cells and right ventricular myocardial hypertrophy are characteristic of MCT pneumotoxicity, we proposed that lung ODC activity would increase prior to development of MCT-induced pulmonary hypertension and right ventricular hypertrophy. To test this hypothesis, we determined lung ODC activity and ODC and AdoMet-DC activities in right and left ventricles during development and progression of MCT-induced pulmonary hypertension and right ventricular hypertrophy.

MATERIALS AND METHODS

Male Sprague–Dawley rats from Harlan Industries were maintained on standard Purina lab chow and water *ad lib.* for at least 10 days prior to initiation of experiments. Animals were housed in wire hanging cages in a room kept at constant temperature with a 6:00 a.m. to 6:00 p.m. photoperiod. The MCT treatment followed the protocol of Hilliker *et al.* [4]; rats (280–320 g) received a single dose of MCT (105 mg/

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kg; s.c.) or normal saline and were killed 1, 4, 7, 10, 14, 16 and 21 days later. At the time of sacrifice rats were anesthetized with sodium pentobarbital (60 mg/kg) and artificially ventilated through a tracheal cannula. After a median sternotomy, a 22 g needle attached to a Statham pressure transducer was inserted through the right ventricle wall and advanced into the pulmonary artery. Pulmonary artery pressure changes were recorded on a Grass polygraph. Mean pulmonary artery pressure was estimated according to the formula: diastolic pressure + (1/3 pulse pressure). Lungs and heart were excised and placed in cold homogenization buffer immediately following determination of pulmonary artery pressure. Whole lungs were cleaned of excess tissue, blotted, weighed and rapidly frozen. Atria were removed and discarded, and the right ventricle (RV) was separated from the left ventricle plus septum (LV + S). The RV and LV + S were separately weighed and rapidly frozen in liquid nitrogen.

Frozen tissues were homogenized in cold buffer A (25 mM Tris–HCl, pH 7.5, 40 μM pyridoxal phosphate, 0.1 mM EDTA and 2.5 mM dithiothreitol) using an Ultra-Turrax homogenizer (full speed, two bursts for 5 sec each). The homogenates were centrifuged at 48,000 *g* for 20 min at 4° and aliquot parts of the resulting supernatant fraction were utilized for ODC, AdoMet-DC and protein determinations. ODC or AdoMet-DC activity was assayed by determining the amount of $^{14}CO_2$ released from 0.5 μCi of L-[1-^{14}C]ornithine or 0.2 μCi of *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine, respectively, during a 60-min incubation at 37°. ODC assay tubes contained 35 μl of the 48,000 g supernatant fraction, buffer A and 0.5 mM L-[1-^{14}C]ornithine in a total volume of 0.20 ml [19]. AdoMet-DC activity was determined by a modification of Pegg [20] in a 0.20-ml assay solution containing 35 μl of the 48,000 *g* supernatant fraction, buffer A, 2.5 mM putrescine and 0.2 mM *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine. The amount of product generated under these assay conditions was proportional to incubation time and the amount of protein. Protein concentration was determined by the method of Bradford [21] using bovine serum albumin as standard.

L-[1-^{14}C]Ornithine (49.6 mCi/mmole) and *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine (52.6 mCi/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). MCT was purchased from Trans World Chemicals, Inc. (Washington, DC), and all other biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Statistical analyses were made using analysis of variance [22].

RESULTS

A single dose of MCT elevated mean pulmonary artery pressure to levels significantly greater than controls at days 16 and 21 (Fig. 1). Right ventricular hypertrophy was evident in MCT-treated rats by day 14 as indicated by the significant increase in the RV/(LV + S) ratio which progressively increased through day 21 (Fig. 2). The wet lung-to-body-weight ratio of the MCT-treated rats was elevated sig-

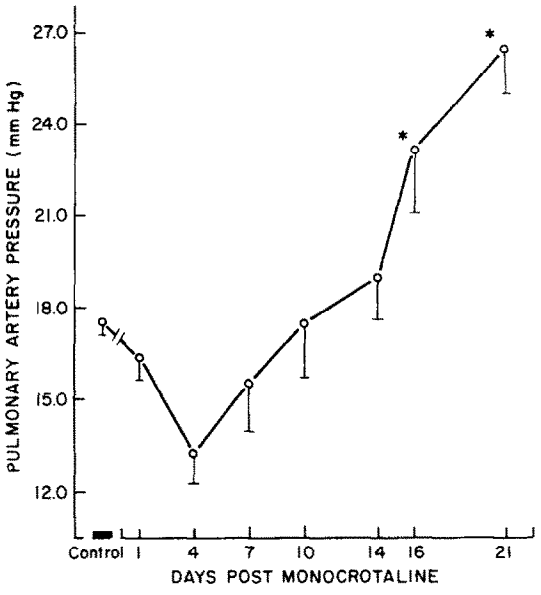

Fig. 1. Changes in pulmonary artery pressure following a single dose of MCT (105 mg/kg, s.c.). Rats were anesthetized at the times indicated following MCT, and pulmonary artery pressure was determined as described in Materials and Methods. The values obtained from animals given 0.9% NaCl (s.c.) are shown as controls and did not change throughout the experiment. Each point represents the mean ± S.E. (bars) for at least nine rats. Astericks indicate data that differ from controls at $P < 0.05$.

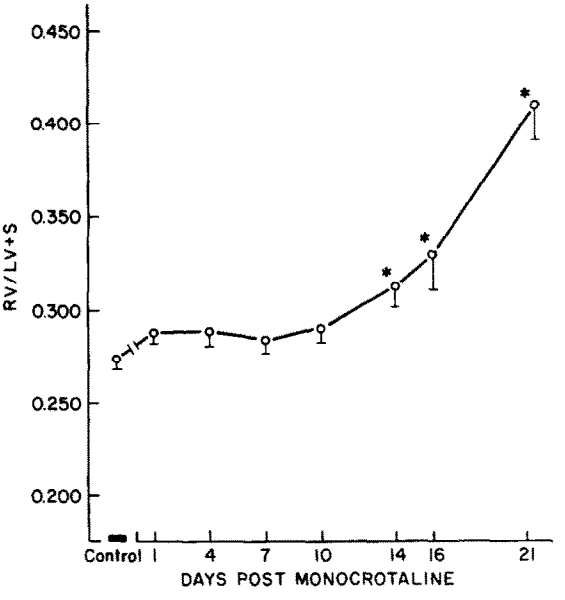

Fig. 2. Changes in the ratio of weight of right ventrical (RV) to left ventricle plus septum (LV + S) following a single dose of MCT (105 mg/kg, s.c.). Rats were killed at the times indicated following MCT, and RV/LV + S was determined as described in Materials and Methods. The values obtained from animals given 0.9% NaCl (s.c.) are shown as controls and did not change throughout the experiment. Each point represents the mean ± S.E. (bars) for at least nine rats. Asterisks indicate data that differ from controls at $P < 0.05$.

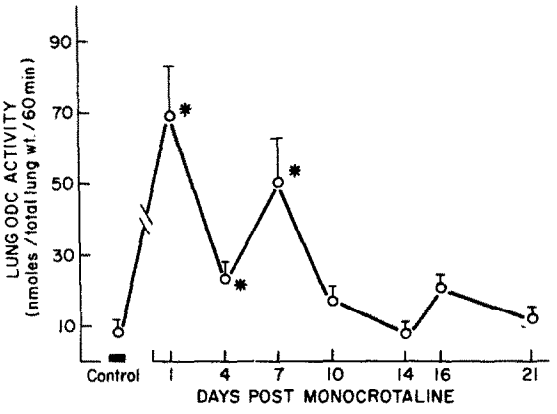

Fig. 3. Changes in lung ODC activity following a single dose of MCT (105 mg/kg, s.c.). Rats were killed at the times indicated following MCT, and ODC activity was determined as described in Materials and Methods. The values obtained from animals given 0.9% NaCl (s.c.) are shown as controls and did not change throughout the experiment. Each point represents the mean ± S.E. (bars) of triplicate determinations of at least nine rats. Asterisks indicate data that differ from controls at $P < 0.05$.

nificantly 7 days post MCT, and this ratio continued to increase to a level 2.5-fold greater than controls by day 21 (data not shown).

An 8-fold increase in lung ODC activity was observed as early as 24 hr following a single injection of MCT (Fig. 3). Seven days after MCT administration, lung ODC activity was still significantly elevated at a level almost 6-fold greater than controls (Fig. 3). Since various lung injuries including that produced by MCT commonly cause increased exogenous protein accumulation in lung [5, 13, 23, 24], ODC activity was normalized to whole lung wet weight rather than expressed per protein content, as has been reported by other investigators [13]. Similar results were obtained when ODC activity was expressed per mg protein.

Table 1. Effects of a single dose of MCT (105 mg/kg, s.c.) on rat cardiac ODC activity

Days post MCT treatment*	No. of rats	Cardiac ODC activity† (pmoles/mg protein/60 min) Right ventricle	Left ventricle
Controls	20	316 ± 29	394 ± 39
1	9	396 ± 35	519 ± 40
4	9	403 ± 59	468 ± 32
7	9	320 ± 49	667 ± 45‡
10	10	395 ± 29	417 ± 44
14	9	361 ± 36	335 ± 36
16	9	200 ± 35	180 ± 23‡
21	12	256 ± 46	202 ± 21‡

* Rats were killed at the times indicated following MCT. The values obtained from animals given 0.9% NaCl (s.c.) are shown as controls and did not change throughout the experiment.

† Mean ± S.E. of triplicate determinations on each rat ventricle.

‡ Data differ from those of control at $P < 0.05$.

Table 2. Effects of a single dose of MCT (105 mg/kg, s.c.) on rat cardiac AdoMet-DC activity

Days post MCT treatment*	No. of rats	Cardiac AdoMet-DC activity† (pmoles/mg protein/60 min) Right ventricle	Left ventricle
Controls	20	50 ± 3	51 ± 4
1	9	53 ± 3	57 ± 3
4	9	70 ± 11	77 ± 8
7	9	81 ± 12‡	111 ± 23‡
10	10	51 ± 6	48 ± 4
14	9	96 ± 23‡	60 ± 12
16	9	45 ± 5	64 ± 6
21	12	27 ± 3	43 ± 4

* Rats were killed at the times indicated following MCT. The values obtained from animals given 0.9% NaCl (s.c.) are shown as controls and did not change throughout the experiment.

† Mean ± S.E. of triplicate determinations on each rat ventricle.

‡ Data differ from those of controls at $P < 0.05$.

Left ventricular ODC activity was increased significantly at day 7; however, it was decreased significantly at days 16 and 21 post MCT (Table 1). Although right ventricular ODC activity was not altered significantly by MCT treatment, there appeared to be a decrease in ODC activity at days 16 and 21 post MCT similar to that observed in the left ventricle. AdoMet-DC activity was transiently elevated in both the right and left ventricles at day 7 following MCT, and only right ventricular AdoMet-DC activity was increased at day 14 (Table 2).

DISCUSSION

MCT-induced lung injury is associated with numerous responses including hypertrophy and proliferation of pulmonary vascular smooth muscle cells [1–5, 25–27] and type II pneumocytes [26, 28]. The early and prolonged increase in lung ODC activity observed in the present study is the first report of the activation by MCT of an enzyme proposed to be involved in the regulation of cell proliferation and differentiation. Furthermore, the activation by MCT of lung ODC for at least 7 days is significant since similar prolonged effects on ODC activity by a single dose of a toxic agent have been reported only for the liver. In those studies the chemical-induced liver cell injury was followed by early and prolonged elevation of liver ODC activity and polyamine levels which preceded and coincided with the development of cell proliferation [19, 29, 30]. Although we did not determine the time course of MCT-induced lung cell proliferation, several other laboratories have shown that proliferation and hypertrophy of lung cells were delayed until 7–12 days after the initiation of MCT dosing [1–3, 25, 27]. It appears, therefore, that early activation of lung ODC by MCT occurred prior to lung cell proliferation. Chemical-induced lung injury and the resulting repair processes have been associated with elevated lung polyamine levels. Increased lung polyamine biosynthesis occurred prior to or during the same time period as increased DNA synthesis, resulting from lung injury caused by hyperoxia

or oxone [13], cadium or chlorphenteramine [14], or a single dose of diethylnitrosamine (J. W. Olson, unpublished observation). Our data suggest the possibility of, but do not conclusively demonstrate, a causal relationship between MCT-induced lung injury, the prolonged activation of ODC, and subsequent cell proliferation that may lead to the increased pulmonary vascular resistance and eventual development of pulmonary hypertension associated with MCT lung toxicity.

The chronological sequence and degree of both right ventricular hypertrophy and increased wet lung-to-body-weight ratios caused by MCT treatment in the present experiments paralleled those reported earlier by Hilliker *et al.* [4]. The right ventricular hypertrophy is believed to be a result of increased pulmonary vascular resistance caused by MCT lung toxicity [1]. The reason why we observed a significant increase in RV/LV + S 2 days before a significant increase in pulmonary artery pressure is not clear but could include the route of administration, dose, or source of MCT; in addition, our relatively insensitive procedure for determining pulmonary artery pressure may not have detected a significant increase in pressure at day 14. Ghodsi and Will [3] did not observe a significant increase in pulmonary artery pressure prior to right ventricular hypertrophy in rats injected with a single dose of MCT (60 mg/kg, s.c.), but rats fed MCT-containing seeds had elevated pulmonary artery pressure at day 14 while right ventricular hypertrophy was not elevated until day 28 [2]. Despite these differences, the important observation of our study is that MCT-induced sustained elevation of lung ODC activity occurred at least 1 week prior to elevated pulmonary artery pressure and right ventricular hypertrophy.

Several studies have demonstrated that cardiac hypertrophy induced by a number of stimuli is associated with increased polyamine biosynthesis [7]. For example, Krelhaus *et al.* [14] reported that exposure of rats to a hypobaric environment (400 mm Hg) resulted in pulmonary hypertension and increased right ventricular ODC activity that preceded the ensuing right ventricular hypertrophy. These results suggested that MCT-induced right ventricular hypertrophy might also be associated with prolonged activation of right ventricular ODC and AdoMet-DC. However, data reported in the present study do not support this relationship. MCT did transiently elevate right ventricular AdoMet-DC activity prior to right ventricular hypertrophy. Interestingly, ODC activity in both right and left ventricles was lowered to a similar degree at days 16 and 21; however, only the decrease in left ventricular ODC activity was stastically significant. The importance of these observations is not clear, although several possibilities may account for our data. First, a significant but transient increase in right ventricular ODC activity could have occurred at time points that were not measured. Second, increased ODC activity and/or a sustained increase in AdoMet-DC activity in the right ventricle may not be essential for development of the cardiac hypertrophy associated with MCT pneumotoxicity. In this context, the maintenance of cardiac polyamines at basal levels may be sufficient for development of left ventricular hypertrophy in some animal models [7, 15, 16]. Our observation that right ventricular ODC activity was not changed significantly at days 16 and 21 while left ventricular ODC activity was decreased significantly at days 16 and 21 could conceivably reflect the balance between two divergent and/or opposing effects of MCT toxicity. One such aspect of MCT toxicity, such as decreased food consumption, could inhibit both right and left ventricular ODC activities while another aspect of MCT toxicity, such as increased pulmonary vascular resistance, could activate ODC and AdoMet-DC only in the right ventricle. It has been reported that a significant suppression of both right and left ventricular ODC activities occurs for as long as rats are required to consume a restricted amount of food [14]; thus it is important to note that MCT-treated rats are known to consume less food and gain less body weight than controls (data not shown, and Ref. 31). The possibility of both inhibiting and activating effects of MCT upon right ventricular ODC activity might actually be reflected by the significant increases in the ratio of right and left ventricular ODC activities at days 14, 16 and 21 ($P < 0.05$, calculated data not shown). Perhaps determination of putrescine and polyamine levels in the right and left ventricles of MCT-treated rats will help clarify this point. It should be emphasized that our data suggest that MCT alters polyamine biosynthesis primarily in the lung, with effects upon the heart apparently occurring secondarily.

In conclusion, these studies present the first evidence for the potential involvement of ODC, a proposed key regulatory protein, in the early development processes of MCT-induced pulmonary hypertension. Additional evidence for a causal relationship between the MCT-caused prolonged elevation of lung ODC activity and increased pulmonary artery pressure would entail the determination of lung polyamine levels and other polyamine biosynthetic regulatory proteins, the cellular localization of lung ODC activity, and the reversal of MCT toxicity with specific inhibitors of polyamine metabolism.

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