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INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS BY DIFLUOROMETHYLORNITHINE

ROLE OF POLYAMINE METABOLISM IN MONOCROTALINE-INDUCED PULMONARY HYPERTENSION

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Abstract—Previously, we have shown that the protection provided by 2-difluoromethylornithine (DFMO) against the development of monocrotaline (MCT)-induced pulmonary hypertension (PH) is associated with inhibition of polyamine biosynthesis in the lungs of MCT-treated rats. Although these studies suggest that prevention of the development of MCT-induced PH is polyamine dependent, no one has demonstrated which cellular events of MCT-induced PH are polyamine dependent. In the present study, using DFMO we tested the hypothesis that inhibition of polyamine biosynthesis may protect against MCT-induced PH by limiting increases in DNA synthesis. We injected rats with MCT (60 mg/kg) or 0.9% NaCl and measured DNA synthesis 7 days after MCT by determining [^{3}H]thymidine incorporation into whole lung DNA. We found that 7 days after MCT treatment DNA synthesis was increased compared to the control (0.9% NaCl). However, DFMO treatment (2% in drinking water) reduced the increase in DNA synthesis following MCT. To confirm that DFMO was acting as a specific inhibitor of polyamine biosynthesis in MCT-induced PH, we administered DFMO concurrently with exogenous ornithine (ORN) (2% in drinking water), the substrate for polyamine biosynthesis, to reverse the protection afforded by DFMO against MCT-induced PH. Twenty-one days after MCT injection we examined right ventricular hypertrophy (RVH), mean pulmonary arterial pressure (MPAP), lung wet weight, and lung polyamine levels. While animals given DFMO (MCT + DFMO) did not increase RVH, MPAP, lung wet weight, or lung polyamine levels, animals given ORN (MCT + DFMO + ORN) did develop increases paralleling those found in animals treated with MCT alone. Our results suggest that suppression of polyamine biosynthesis by DFMO may protect against the development of MCT-induced PH in part by preventing increases in DNA synthesis. This suppression of DNA synthesis may limit the proliferation of key lung cells involved in the inappropriate vascular remodelling associated with MCT-induced PH. These results are consistent with our working hypothesis that elevated lung polyamine levels are essential for the development of MCT-induced PH.

Monocrotaline (MCT)† is a pyrrolizidine alkaloid found in plants [1]. Rats given a single dose of MCT develop pulmonary hypertensive vascular disease with pulmonary arterial medial thickening and right ventricular hypertrophy (RVH), resembling human primary pulmonary hypertension [2–7]. The endothelium appears to be the initial site of injury [6, 8, 9] followed by the appearance of pulmonary edema, inflammation, vascular hypertrophy and hyperplasia, and increased vascular resistance. These early changes are followed by vascular remodelling which is associated with sustained pulmonary hypertension (PH) and RVH [6, 8, 9]. Despite numerous studies the biochemical mechanisms involved in MCT toxicity are uncertain.

Several approaches have been tried in an effort to protect animals from the development of MCT-induced PH. These approaches include hyperoxia, methylprednisolone, and thrombocytopenia [7, 10, 11]. In each of these studies the authors have reported a reduction in RVH and/or mean pulmonary arterial pressure (MPAP) associated with the development of MCT-induced PH. The only report [12] in which increases of MPAP and RVH were prevented is when polyamine biosynthesis was blocked by the administration of 2-difluoromethylornithine (DFMO), a specific inhibitor of polyamine biosynthesis [13, 14].

The diamine putrescine and the polyamines spermidine and spermine are ubiquitous and have been shown to be essential for growth and differentiation of both normal and neoplastic tissue [15–17]. The administration of DFMO, a competitive inhibitor of ornithine decarboxylase (ODC) (EC 4.1.1.17), protects against MCT-induced PH by preventing the increases in both MPAP and medial thickness and RVH [18]. This protection provided by DFMO against MCT-induced PH is associated with limiting increases in lung polyamine biosynthesis

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† Abbreviations: MCT, monocrotaline; DFMO, 2-difluoromethylornithine; ODC, ornithine decarboxylase; AdoMet-DC, *S*-adenosylmethionine decarboxylase; RV, right ventricle; LV + S, left ventricle plus septum; MPAP, mean pulmonary arterial pressure; PH, pulmonary hypertension; RVH, right ventricular hypertrophy; ORN, ornithine; and TPA, 12-*O*-tetradecanoylphorbol-13 acetate.

normally associated with MCT pneumotoxicity and suggests that prevention of PH is polyamine dependent. Although DFMO protects against the development of MCT-induced PH and this protection is associated with a reduction of polyamine levels, no one has elucidated the mechanism(s) by which limiting increased polyamine biosynthesis provides protection against the development of MCT-induced PH.

In the present study, using DFMO, we tested the hypothesis that inhibition of polyamine biosynthesis may protect against MCT-induced PH by limiting increases in DNA synthesis associated with the development of MCT-induced PH. To confirm that DFMO was acting as a specific inhibitor of polyamine biosynthesis in MCT-induced PH, we administered DFMO concurrently with exogenous ornithine (ORN), the substrate for polyamine biosynthesis, to reverse the protection afforded by DFMO against MCT-induced PH. Ornithine has been shown to protect ODC from inactivation by preventing the binding of DFMO to its receptor *in vitro* [13, 19]. The administration of exogenous ORN can inhibit or prevent DFMO from binding to ODC because ORN is actively transported into cells [20] leading to increases in intracellular ORN concentration, while DFMO enters cells by passive diffusion [21].

METHODS

Male Sprague–Dawley rats (50 days old weighing 200–230 g, specific pathogen free) from Hilltop Laboratories (Scottsdale, PA) were used. They were divided into control and treatment groups (five animals per group). All were housed in individual cages and maintained in a laminar flow animal isolation unit at a constant temperature with a 12-hr photoperiod. Daily measurements were made of body weight, water and food intake. The MCT treatment used was as previously reported [12]. Animals were given a single dose of MCT (60 mg/kg, s.c.) or 0.9% NaCl and killed 7 or 21 days later. DFMO and ORN treatments (2% each in the drinking water) were begun 4 days before MCT. On the day of MCT administration animals receiving DFMO were given injections of DFMO (400 mg/kg, s.c.) 2 hr before MCT and at 2, 4, and 8 hr following MCT [12]. When the animals were killed, they were anesthetized with sodium pentabarbital (60 mg/kg) and a medial sternotomy was performed. The animals were ventilated mechanically through a tracheal cannula. A 22-gauge needle attached to a Statham P 23XL pressure transducer was inserted through the right ventricle wall and positioned in the pulmonary artery. Changes in pulmonary arterial pressure were recorded on a Unigraph recorder (Gilson). Mean pulmonary arterial pressure was computed using the formula: diastolic pressure + 1/3 pulse pressure [22]. The lungs and heart were removed and placed in 0.9% NaCl at 4°. The lungs were then homogenized in a solution consisting of 48 mM sodium phosphate buffer (pH 7.2), 0.1 mM pyridoxal 5′-phosphate, 5 mM dithiothreitol, and 0.1 mM EDTA at 4° for 30 sec with a Tissumizer (Tekmar). The volume was adjusted to 5.0 mL. Aliquots were taken for protein, DNA and polyamine determinations. The atria were dissected free from the heart, and the right ventricle (RV) was isolated from the left ventricle plus septum (LV + S). The RV and the LV + S were weighed and their ratio was used to estimate the extent of RVH [23].

Lung polyamine levels were determined as reported previously by Hacker and coworkers [24, 25] using an extraction procedure similar to that of Seiler and Weichmann [26]. A 0.5-mL aliquot of lung homogenate was mixed with 0.5 mL of 0.4 N perchloric acid, centrifuged at 30,000 *g* for 10 min at 4°, and the supernatant was collected. A 0.2-mL aliquot of the supernatant was combined with 0.6 mL of 0.28 M dansyl chloride in acetone:water (7:3, v/v). The pH of each sample was adjusted to 9.8 to 10.2 using a solution of 0.6 M sodium bicarbonate at pH 9.7. Five standards and two blanks were also derivatized. All tubes were placed in the dark for 18 hr. After 18 hr, 0.2 mL of 1.3 M proline was added to each tube to remove excess dansyl chloride. Dansylated polyamines were extracted into 6 mL of ethyl acetate. The ethyl acetate was collected and evaporated to dryness under nitrogen at 40°. The final extract was dissolved in 1 mL of ethyl acetate, and 0.02 mL was injected into a high performance liquid chromatograph (Varian 5020 Fluorochrom fluorescence detector, excitation at 360 and emission at 440 nm). The polyamines were separated using a reverse-phase column (Varian MCH-10, C_{18} 10 μm silica) with a gradient of water:acetonitrile beginning at 50:50 and ending with 5:95. A 3-cm guard column was used to protect the MCH column. Values were corrected for recovery using 1.6 diaminohexane as an internal standard. DNA synthesis was determined using a separate group of animals 7 days post MCT. Fifteen animals were divided randomly into three treatment groups (MCT, MCT + DFMO, or control) of five animals each. DNA was estimated by the incorporation of radioactivity into DNA after injecting 1 μCi/g body weight of [*methyl*$^{-3}$H]-thymidine (Amersham) intraperitoneally [27]. The DNA was extracted as follows: 1 hr after injection of [*methyl*$^{-3}$H]thymidine the animals were anesthetized as described above, and the lungs were perfused with 0.9% sodium chloride; hilar tissue was removed, and the lungs were homogenized in distilled water as above. An appropriate aliquot was combined with an equal volume of 0.4 N perchloric acid (4°) mixed and centrifuged at 30,000 *g* for 20 min. The supernatant was discarded, and the procedure was repeated. The pellet was extracted twice with ethanol at room temperature and centrifuged as above. The resulting pellet was extracted with 0.2 N perchloric acid at 90° for 15 min. The supernatant was collected and used for measurement of [^{3}H]thymidine incorporation and DNA content. Tritiated thymidine incorporation was measured by counting an appropriate aliquot in a Beckman liquid scintillation counter (Beckman Instruments, Fullerton, CA) using an external standard. Incorporation into lung DNA is reported as dpm/μg DNA. DNA and protein content were determined by the methods of Schneider [28] and Lowry *et al.* [29] respectively.

Data are expressed as the mean ± SEM. Differences between experimental groups were examined using analysis of variance (ANOVA) and

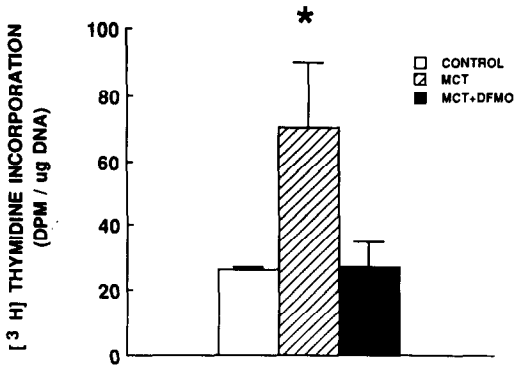

Fig. 1. Changes in the incorporation of [^{3}H]thymidine into lung DNA following MCT and MCT + DFMO. Control rats received 0.9% NaCl (s.c.). All groups were killed 7 days post MCT 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates a value significantly different from control at $P < 0.05$.

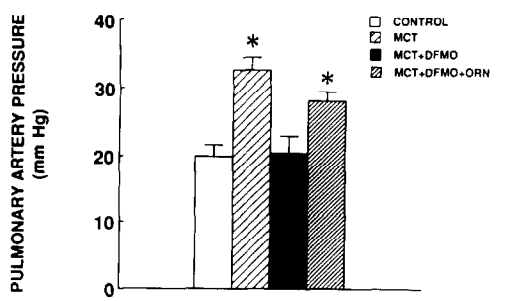

Fig. 2. Changes in MPAP following MCT, MCT + DFMO and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates values significantly different from control at $P < 0.05$.

Dunnett's test for multiple comparisons. A P value equal to or less than 0.05 was considered statistically significant. MCT was purchased from Trans World Chemicals (Washington, DC). DFMO (MDL 71,782) was a gift from Merrell Dow Research Institute, Cincinnati, OH. All other chemicals were reagent grade.

RESULTS

Preliminary studies in our laboratory demonstrated that treatment of rats with MCT (60 mg/kg) resulted in increased [^{3}H]thymidine incorporation into whole lung DNA that was maximal at 7 days. As shown in Fig. 1, [^{3}H]thymidine incorporation into whole lung DNA was increased more than 2-fold above control 7 days after a single injection of MCT. Treatment with DFMO (MCT + DFMO) prevented increases in [^{3}H]thymidine incorporation into whole lung DNA associated with MCT.

To confirm that DFMO was acting as a specific inhibitor of polyamine biosynthesis in MCT-induced PH we gave exogenous ORN, the substrate for polyamine biosynthesis, to reverse the inhibitory effect of DFMO upon lung polyamine biosynthesis 21 days post MCT. We chose 21 days because evidence of sustained PH (increases in mean pulmonary artery pressure and right ventricular hypertrophy) is not detectable until 14–21 days post MCT injection. We found that MPAP was elevated above control in animals treated with MCT ($P < 0.05$) (Fig. 2). Treatment with DFMO (MCT + DFMO) prevented increases in MPAP associated with MCT. Mean pulmonary arterial pressure was increased above control ($P < 0.05$) following administration of ORN (MCT + DFMO + ORN) resulting in a reversal of the protection provided by DFMO (MCT + DFMO) against MCT-induced PH. Consistent with the above, RV/(LV + S) had increased above control following MCT ($P < 0.05$) and administration of DFMO prevented the increase, while coadministration of ORN (MCT + DFMO + ORN)

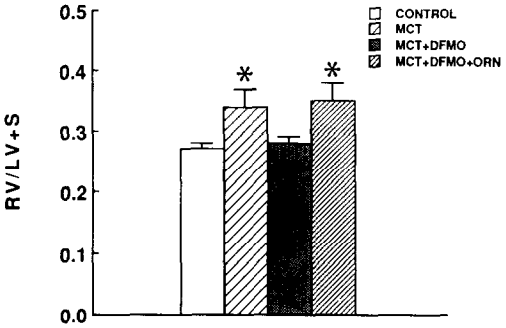

Fig. 3. Changes in the ratio of RV to LV + S following MCT, MCT + DFMO and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates values significantly different from control at $P < 0.05$.

resulted in increased RV/(LV + S) reversing the protection provided by DFMO (Fig. 3). Although lung weight increased following MCT ($P < 0.05$), DFMO prevented the increase. With ORN supplementation, however, lung weight again increased above control resulting in a reversal of the protection provided by DFMO (Fig. 4). In animals given exogenous ORN (MCT + DFMO + ORN) MPAP, RVH and lung weight were not significantly different from MCT alone ($P > 0.05$) (Figs. 2–4).

Consistent with our previous reports [12, 18], lung levels of putrescine, spermidine and spermine increased above control following MCT ($P < 0.05$) (Figs. 5 and 6). Levels of putrescine, spermidine and spermine in the lungs of animals treated with MCT + DFMO were not significantly different from control ($P > 0.05$). Continuous coadministration of ORN and DFMO caused increases of putrescine, spermidine and spermine above control ($P < 0.05$), resulting in reversal of the inhibition of the biosynthesis of these polyamines in the lung by

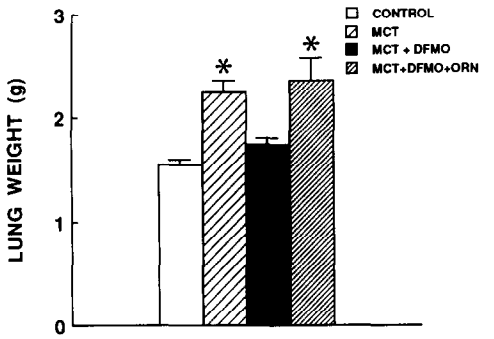

Fig. 4. Changes in lung wet weight (g) following MCT, MCT + DFMO and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates values significantly different from control at $P < 0.05$.

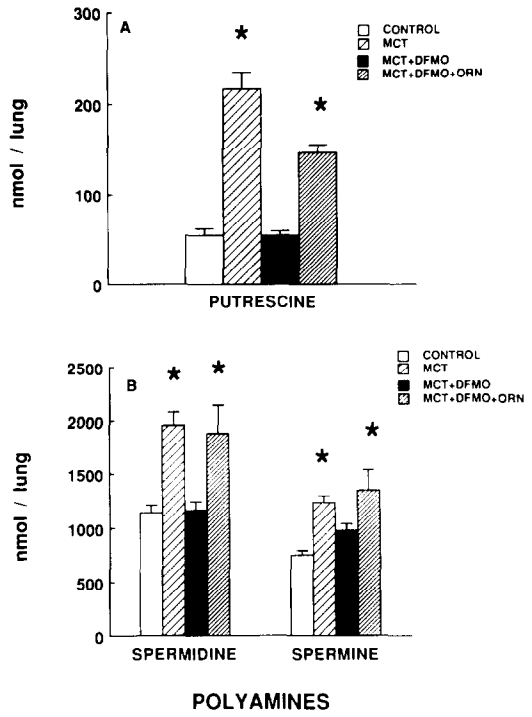

Fig. 5. Changes in the polyamine contents per lung following MCT, MCT + DFMO, and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates values significantly different from control at $P < 0.05$.

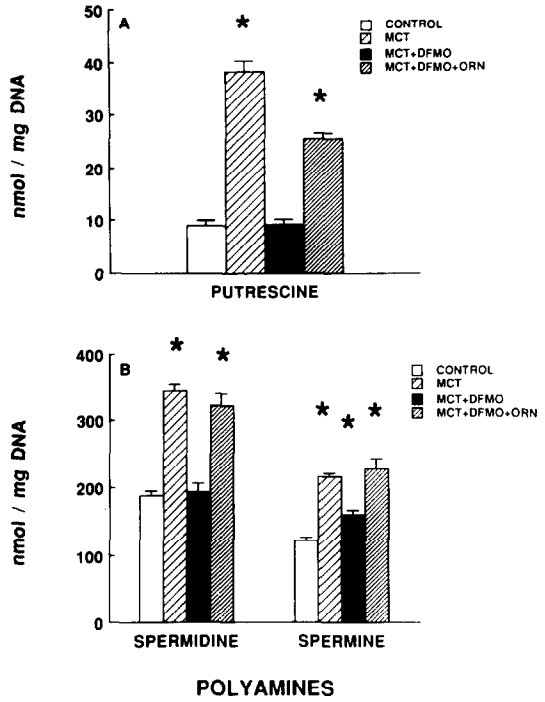

Fig. 6. Changes in the polyamine contents per mg DNA following MCT, MCT + DFMO, and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group. An asterisk (*) indicates values significantly different from control at $P < 0.05$.

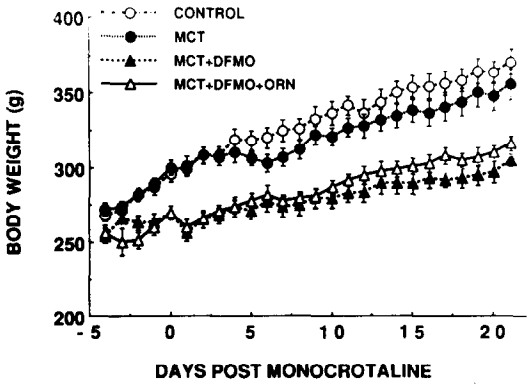

Fig. 7. Changes in body weight following MCT, MCT + DFMO, and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group.

DFMO. Similar results were obtained when polyamines were expressed per lung or per mg DNA.

Animals in the MCT + DFMO and MCT + DFMO + ORN groups were given water containing 2% DFMO and 2% DFMO + 2% ORN, respectively, 4 days prior to MCT injection on day 0. This resulted in an inhibition of growth as indicated in Fig. 7 where the body weight of rats in groups MCT + DFMO and MCT + DFMO + ORN remained relatively unchanged or decreased prior to MCT

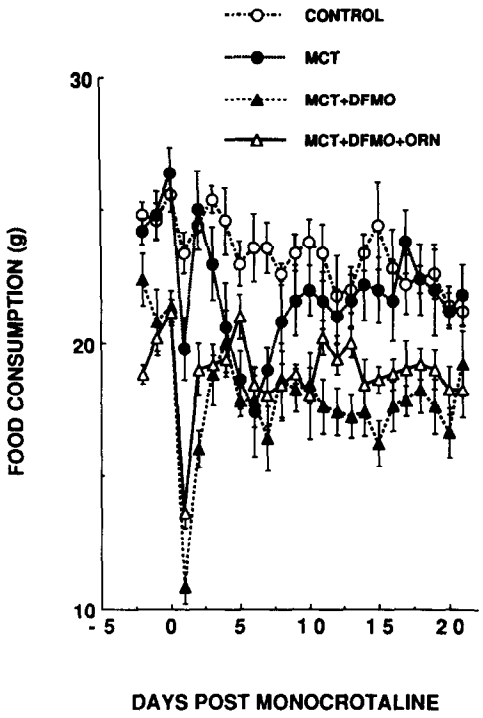

Fig. 8. Changes in food consumption following MCT, MCT + DFMO, and MCT + DFMO + ORN. Control rats received 0.9% NaCl (s.c.). All groups were killed 21 days post MCT or 0.9% NaCl. Values are means ± SEM; N = 5 for each group.

injection. This is in contrast to animals in the control or MCT groups where the body weight continued to increase. The reduced growth rate of the MCT + DFMO and MCT + DFMO + ORN groups was associated with a decrease in food consumption (Fig. 8) compared to animals in the MCT and control groups. Twenty-four hours after injection of MCT the body weight of animals in the MCT + DFMO and MCT + DFMO + ORN groups decreased. This was associated with a dramatic fall in food consumption from 21 to 11 g in the MCT + DFMO group and from 21 to 14 g in the MCT + DFMO + ORN group. A decrease was also observed in the control and MCT groups 24 hr post MCT injection but of a lesser degree. Animals in the MCT group exhibited a second decrease in food intake from 4 to 7 days, a time during which edema has been reported [6, 30]. A similar decline was observed for animals in the MCT + DFMO and MCT + DFMO + ORN groups but of lesser magnitude. Animals in all groups continued to grow through 21 days post MCT. From 6 to 21 days post MCT the control group displayed the highest rate of growth, while the MCT group lagged approximately 5% behind. The body weight of animals in the MCT + DFMO and MCT + DFMO + ORN groups remained approximately 15% less than controls during the same period.

DISCUSSION

The mechanism(s) responsible for the toxic effects of MCT is unknown. Previous reports [31, 32] have indicated that MCT is biotransformed in the liver by cytochrome P450 to toxic metabolites which can damage pulmonary artery endothelial cells [32, 33]. Although this mechanism is commonly proposed for MCT toxicity in the lung, we have preliminary evidence [34] that endothelial cells are capable of biotransformation of MCT and may become injured without hepatic metabolism of MCT.

Cell proliferation may be an important factor particularly during the first 7 days of lung injury associated with MCT toxicity [12]. Arterial cell proliferation and hypertrophy may play an important role in the development of MCT-induced PH. Rats fed *Crotalaria spectabilis* seeds developed MCT-induced PH and had hyperplasia and hypertrophy of pulmonary arterial cells at pre- and intra-acinar levels [35]. Changes in the labeling index varied with cell types and were observed in smooth muscle, fibroblast and endothelial cells from 3 to 35 days. The authors suggested that the early increase in labeling indexes at 3 and 7 days probably represents a response to injury. These changes in cell proliferation occur before increases in medial or adventitial thickness or pulmonary artery pressure [35]. In our previous studies with MCT we found that the timing of increased polyamine biosynthesis precedes the development of RVH and elevated pulmonary artery pressure [12]. This increase appears to correlate with repair of MCT-induced pulmonary arterial injury as reflected by increased cell proliferation [35].

The polyamines, putrescine, spermidine and spermine, are involved with the regulation of cell growth and differentiation [36–38]. Ornithine decarboxylase is the initial and a rate-limiting enzyme in polyamine biosynthesis [16, 36–38], while *S*-adenosylmethionine decarboxylase (AdoMet-DC) (EC 4.1.1.50) is the rate-limiting enzyme for the conversion of putrescine to spermidine and spermidine to spermine [36–38]. We have found that continuous administration of DFMO, a highly specific enzyme-activated, irreversible inhibitor of ODC activity [13] prevents the development of MCT-induced PH and RVH [12]. These observations suggest that prolonged elevation of lung polyamine concentrations may be essential for the development of hypertensive vascular disease associated with MCT toxicity, but the molecular events altered by DEMO are unknown.

In the present study, using DFMO, we tested the hypothesis that inhibition of polyamine biosynthesis may protect against MCT-induced PH by limiting increases in DNA synthesis associated with the development of MCT-induced PH. Although the requirement of polyamines for DNA replication and cell proliferation is established, no such role in MCT-induced PH has been reported.

Evidence for a role of polyamines in the regulation of DNA synthesis is abundant. Studies using mutation or deletion in genes coding for the polyamine biosynthetic enzymes as well as those using specific inhibitors, such as DFMO, have shown that increases of cellular polyamine levels are a requirement, not a consequence, of cell growth and proliferation [39]. The drug DFMO has been shown

to inhibit the synthesis of DNA in a variety of normal and neoplastic cells and tissues [17]. Luk [40] found that DFMO inhibits polyamine and DNA synthesis during liver regeneration following partial hepatectomy. A recent report by Byus and Wu [41] demonstrated that insulin- and 12-*O*-tetradecanoylphorbol-13 acetate (TPA)-induced DNA synthesis in Reuber H35 hepatoma cells was dependent upon polyamine biosynthesis.

The amino acid ornithine is the precursor for polyamine biosynthesis. It is an intermediate in the urea cycle and is not incorporated into protein [42]. Ornithine is not present in the diet but is derived from the hydrolysis of arginine. Unlike DFMO, which appears to enter cells by passive diffusion [21], ORN is transported actively [20]. Ornithine has been shown to protect ODC from inactivation by preventing the binding of DFMO to its receptor *in vitro* [13, 19]. To confirm that DFMO was acting as a specific inhibitor of polyamine biosynthesis in MCT-induced PH, we administered DFMO concurrently with exogenous ORN, the substrate for polyamine biosynthesis, to reverse the protection afforded by DFMO against MCT-induced PH. We found that ORN treatment resulted in increased levels of lung polyamines paralleling those found in animals treated with MCT (60 mg/kg) alone. Ornithine treatment reversed the protection provided by DFMO against the development of MCT-induced toxicity as demonstrated by the development of increased MPAP (Fig. 2), RVH (Fig. 3), and lung weight (Fig. 4).

Recently Olson *et al.* [43] reported that delayed administration of DFMO to MCT-treated animals decreases lung polyamine levels and RV/LV + S, and provides partial protection to them from MCT. The effect of delayed administration of DFMO upon sustained PH in MCT-treated animals is uncertain, as the ratio RV/LV + S is an indirect indicator of sustained PH; determining mean pulmonary artery pressure would have provided a more sensitive measurement. The authors found that simultaneous administration of ORN with DFMO reverses the partial protection against RVH and elevates lung polyamine levels to those found in animals given a low dose of MCT (30 mg/kg) alone.

Total caloric restriction to 45% of usual food intake protects rats from MCT-induced RVH, prolongs survival and is associated with a suppression of growth [44]. This protective effect is not due to differences in the bioactivation of MCT to the monocrotaline pyrrole [45]. We found that DFMO treatment resulted in suppression of growth (Fig. 7). However, when ORN reversed DFMO inhibition of MCT toxicity, growth remained suppressed. These results demonstrate that the protective effect of DFMO against MCT-induced PH is independent of secondary effects upon growth.

Although this study and that of Meyrick and Reid [35] demonstrate that increased DNA synthesis occurs following MCT, it is still unknown whether the cells with increased DNA synthesis go on to divide. Wilson and Segall [46] have reported enlarged type II alveolar epithelial cells in the lungs of rats following exposure to MCT. This observation is similar to reports of enlarged hepatocytes following exposure to pyrrolizidine alkaloids [47].

The mechanism by which DFMO protects against the development of MCT-induced PH is unknown. However, our results suggest that suppression of polyamine biosynthesis by DFMO may protect against the development of MCT-induced PH in part by preventing increases in DNA synthesis. Further, this study demonstrates that the protective effect of DFMO was not due to nonspecific effects upon growth. The present study does not allow us to determine in which cell types DNA synthesis is inhibited by DFMO. Further studies utilizing autoradiography will be required to identify which cells are the key lung cells and to what extent hypertrophy and cell division play a role in the response of lung cells to MCT. These data are consistent with our working hypothesis that increased lung polyamine biosynthesis is required for the development of MCT-induced PH.

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