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DIETARY RESTRICTION, POLYAMINES AND MONOCROTALINE-INDUCED PULMONARY HYPERTENSION

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Abstract—Dietary restriction (DR), i.e. reduction of total caloric intake, has been shown to result in protection against monocrotaline (MCT)-induced pulmonary hypertension (PH). Restriction of the diet to 8 g/rat/day instead of the usual intake (18 g/rat/day), inhibits the progression of cardiopulmonary changes and prolongs survival after a single dose of MCT. We have shown previously that the development of MCT-induced pulmonary hypertension is associated with inhibition of polyamine biosynthesis in the lungs of MCT-treated rats. In the present study, we tested the hypothesis that DR provides protection against the development of chronic PH in the rat by limiting increases in polyamine and DNA synthesis. We randomly divided animals into four groups each (MCT, MCT + DR, control, and control + DR). We injected rats with a single dose of MCT (60 mg/kg, s.c.) and a corresponding number of control rats with vehicle. Animals in all groups were given free access to food and water prior to administration of MCT. Immediately following injection of MCT both the MCT and control groups were given free access to food and water, while the other groups (MCT + DR and control + DR) were given the restricted diet (8 g/rat/day). Daily measurements were made of body weight and of water and food intake. Animals were killed in each group at 1, 4, 7, 14, and 21 days post MCT to determine right ventricular hypertrophy (RVH), lung wet weight, ornithine decarboxylase (ODC) activity, and polyamine and DNA contents. We measured DNA synthesis 7 days after MCT by determining [^{3}H]thymidine incorporation into the whole lung DNA. We found that 7 days after MCT treatment DNA synthesis increased compared to control. However, DR (MCT + DR) treatment prevented the increase in DNA synthesis following MCT. Right ventricular hypertrophy, lung wet weight, ODC activity and lung polyamine levels were increased following MCT. Treatment with DR (MCT + DR) prevented increases in RVH, lung wet weight, ODC activity and lung polyamine levels. We conclude that DR to 8 g/day/rat protects against MCT-induced PH and is associated with an inhibition of increased lung polyamine and DNA synthesis that occur in the lung during the development of MCT-induced PH. These results are consistent with a recent report which suggests that increased lung polyamine biosynthesis is required for the development of MCT-induced PH. The data are also consistent with the hypothesis that inhibition of polyamine biosynthesis influences the development of MCT-induced PH in part by regulating DNA synthesis in key lung cells.

Although a diagnosis of pulmonary hypertension (PH†) can be confirmed by cardiac catherization, our understanding of the basic mechanisms underlying the disease is lacking. However, studies in experimental animals have contributed much to our current knowledge. Chronic PH has been produced using a variety of experimental animal models. These models include increasing blood flow through the pulmonary vascular bed (hyperkinetic), restriction or obstruction of the pulmonary vascular bed, constriction of pulmonary veins, dietary factors, chronic hypoxia, chronic hypercapnia, and some models of lung diseases such as experimental emphysema and silicosis [1].

Chronic lung injury can be accompanied by chronic PH in humans and in animals [2]. Monocrotaline (MCT), a plant pyrrolizidine alkaloid, when administered to rats results in pulmonary vascular injury. After a single dose, pulmonary arterial pressure is elevated and right ventricular hypertrophy (RVH) develops [3–7]. Because of its toxic properties, MCT has been used to study the development of chronic PH [3–7], although the mechanism of its action remains unknown.

Dietary restriction (DR), i.e. reduction of total caloric intake, has been shown to result in protection against MCT-induced PH [8]. Restriction of the diet to 8 g/rat/day instead of the usual intake (18 g/rat/day), inhibits the progression of cardiopulmonary changes and prolongs survival after a single dose of MCT. Animals given MCT and placed on an *ad lib.* diet develop RVH as judged by a cross-sectional area of ventricles and septum. In MCT-treated diet-reduced animals, RVH did not occur. In addition,

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† Abbreviations: PH, pulmonary hypertension; MCT, monocrotaline; DR, dietary restriction; DFMO, 2-difluoromethylornithine; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; RV, right ventricle; LV + S, left ventricle plus septum; RVH, right ventricular hypertrophy β-; and MCTP, monocrotaline pyrrole.

DR also enhances survival [8]. The authors suggest that the effects may be related primarily to reduced food intake or to suppression of growth. In other studies DR decreased polyamine biosynthesis in organs other than the lung [9–13]. Increased polyamine biosynthesis has been reported to be an early event in cells given a stimulus for growth, including partial hepatectomy [14], growth hormones [15, 16] and oxygen toxicity [17–20]. Polyamines are small nitrogen-containing polycationic compounds that appear to influence the regulation of protein synthesis and cell division [21–23]. The enzyme ornithine decarboxylase (ODC, EC 4.1.1.17) catalyses the conversion of ornithine to putrescine, the rate-limiting step in the synthesis of polyamines, and ODC has a half-life of approximately 10–15 min [24].

Increases in ODC activity have been reported in the lung following a single injection of MCT [25, 26]. This increased activity was observed to occur before increases in RVH or pulmonary artery pressure, reflecting PH, could be detected.

We have found that two models of chronic PH (MCT- and hypoxia-induced) result in increases of lung polyamine biosynthesis prior to the development of PH in the rat and that prevention of increased polyamine biosynthesis can protect against MCT-induced PH in the rat [25–28]. These data suggest that increased lung polyamine biosynthesis may be essential for the development of chronic PH in the rat. In this study we tested the hypothesis that DR provides protection against the development of chronic PH in the rat by limiting increases in polyamine and DNA synthesis.

METHODS

Male Sprague–Dawley rats (5 weeks old, specific pathogen free) from Hilltop Laboratories (Scottsdale, PA) 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 and of water and food intake. To determine if DR prevents the increase in polyamine levels associated with the development of MCT-induced PH [25] we randomly divided 100 rats into four groups each (MCT, MCT + DR, control, and control + DR). We injected 50 rats with a single dose of MCT (60 mg/kg, s.c.) and a corresponding number of control rats with vehicle. MCT (200 mg) was dissolved in 0.6 mL of 1 N HCl, neutralized with NaOH, and adjusted to a volume of 10 mL with distilled water as previously described [8]. Animals in all groups were given free access to food and water prior to administration of MCT. Immediately following injection of MCT both the MCT and control groups were given free access to food and water, whereas the other groups (MCT + DR and control + DR) were given the restricted diet (8 g/rat/day) [8]. Daily measurements were made of body weight and of water and food intake. Five animals were killed in each group at 1, 4, 7, 14, and 21 days post MCT to determine RVH, lung wet weight, ODC activity, and polyamine and DNA contents.

Animals were euthanized with sodium pentobarbital (60 mg/kg), and a medial sternotomy was performed. The lungs and heart were removed and placed in 0.9% NaCl at 4°. The lungs were weighed and homogenized for 30 sec with a Tissumizer (Tekmar) 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°. The volume was adjusted to 5.0 mL. Aliquots of the homogenate were taken for polyamine and DNA determinations, and the remainder of the homogenate was centrifuged at 30,000 *g* for 30 min at 4°. The activity of ODC was determined as previously reported [17]. 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 [29].

Lung polyamine levels were determined as reported previously by Hacker and coworkers [17, 20] using an extraction procedure similar to that of Seiler and Weichmann [30]. 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 nm 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 4-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. Sixteen animals were randomly divided into four treatment groups (MCT, MCT + DR, control, and control + DR) of four 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 [31]. 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 perfused with 0.9% sodium chloride; hilar tissue 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

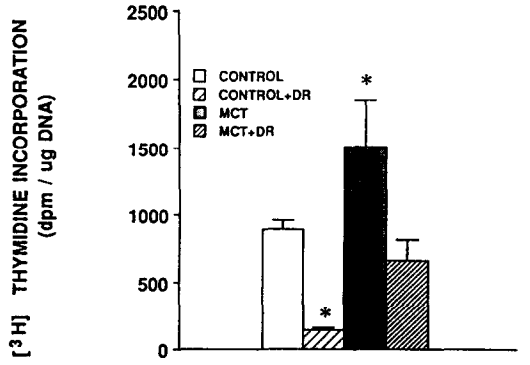

Fig. 1. Changes in the incorporation of [^{3}H]thymidine into lung DNA following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). All groups were killed 7 days post MCT or vehicle. Values are means ± SEM, N = 4 for each group. Key: (*) significantly different from control at $P < 0.05$.

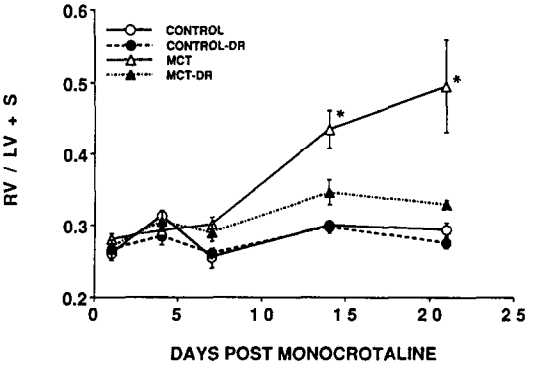

Fig. 2. Changes in the ratio of RV to LV + S following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

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 content was determined by the method of Schneider [32].

Data are expressed as means ± SEM. Differences between experimental groups were examined using analysis of variance (ANOVA) and Scheffe'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). All other chemicals were reagent grade.

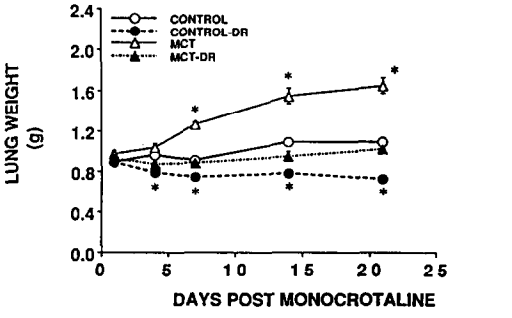

Fig. 3. Changes in the lung wet weight (g) following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

RESULTS

Preliminary studies in our laboratory demonstrated that treatment of rats with MCT (60 mg/kg, s.c.) resulted in increased [^{3}H]thymidine incorporation into whole lung DNA that was apparent at 4 days and reached a maximum at 7 days. As shown in Fig. 1, [^{3}H]thymidine incorporation into whole lung DNA was increased approximately 2-fold above control 7 days after a single injection of MCT ($P < 0.05$). Treatment with DR (MCT + DR) prevented increases in [^{3}H]thymidine incorporation into whole lung DNA associated with MCT. Animals given vehicle and placed on DR (control + DR) exhibited a decrease in [^{3}H]thymidine incorporation into whole lung DNA that was below control ($P < 0.05$).

To determine if DR protects against MCT-induced PH, we measured the weight of the RV/LV + S to estimate right ventricular hypertrophy. We found that RV/LV + S was elevated above control in animals treated with MCT ($P < 0.05$) at 14 and 21 days post MCT (Fig. 2). Treatment with DR (MCT + DR) prevented increases in RV/LV + S associated with MCT. Animals given vehicle and DR (control + DR) were not different from vehicle alone (control, $P > 0.05$). Lung weight increased following MCT ($P < 0.05$) at 7, 14, and 21 days (Fig. 3). Treatment of animals with DR (MCT + DR) prevented increases in lung weight associated with MCT. Animals given vehicle and DR (control + DR) had lung weights that were less than vehicle alone (control, $P < 0.05$) at 4, 7, 14, and 21 days.

To determine if DR prevents the increase in polyamine metabolism associated with the development of MCT-induced PH, we measured the activity of ODC and the contents of polyamines in the lungs. We found that the activity of ODC was increased above control at 4 (8-fold) and 7 (3-fold)

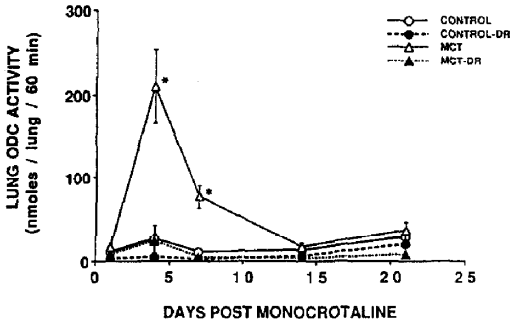

Fig. 4. Changes in the activity of ODC per lung following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

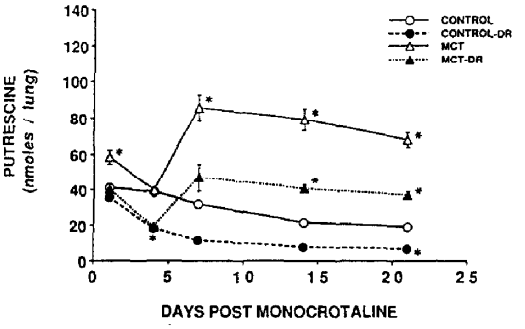

Fig. 6. Changes in the lung putrescine content following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

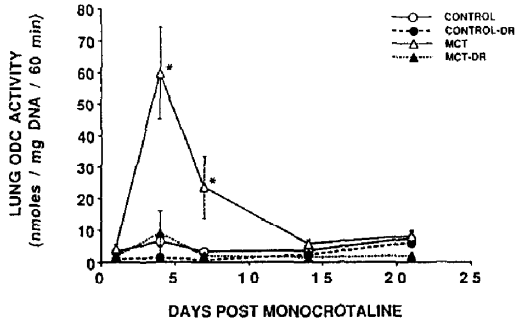

Fig. 5. Changes in the activity of ODC per milligram DNA following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

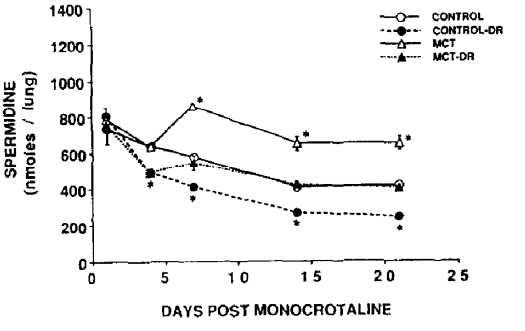

Fig. 7. Changes in the lung spermidine content following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

days following MCT ($P < 0.05$) per lung (Fig. 4). A similar elevation in ODC activity was obtained at 4 (12-fold) and 7 (5-fold) days when ODC activity was expressed per milligram DNA ($P < 0.05$) (Fig. 5). Treatment with DR (MCT + DR) prevented increases in ODC activity (Figs. 4 and 5). Animals given vehicle and DR (control + DR) were not different from vehicle alone (control, $P > 0.05$).

Consistent with our previous reports [25, 26, 33] lung levels of putrescine increased above control following MCT. The level of putrescine increased at 1, 7, 14, and 21 days following MCT ($P < 0.05$) (Fig. 6). In animals treated with MCT + DR, putrescine was not significantly different from control ($P > 0.05$) at 1 and 7 days. Putrescine (MCT + DR) was decreased below control at 4 days ($P < 0.05$) and increased slightly above control at 14 and 21 days ($P < 0.05$) post DR (MCT + DR). In animals given vehicle and DR (control + DR), putrescine was decreased below control at 4 and 21 days ($P < 0.05$).

Following MCT, the levels of spermidine and spermine were increased above control at 7, 14 and 21 days ($P < 0.05$) (Figs. 7 and 8). Levels of spermidine and spermine in the lungs of animals treated with MCT + DR were not significantly different from control ($P > 0.05$) at 7, 14, and 21 days. Spermidine and spermine decreased below control ($P < 0.05$) at 4 days post MCT + DR. In animals given vehicle and DR (control + DR) spermidine was decreased below control at 4, 7, 14, and 21 days, whereas spermine was decreased at 4, 14, and 21 days. Similar results were obtained when polyamines were expressed per lung or per mg DNA.

Animals in the MCT + DR and control + DR groups were given a restricted diet (8 g/rat/day) immediately following MCT or vehicle injection on day 0. This resulted in an inhibition of growth as indicated in Fig. 9 where the body weight of rats in groups MCT + DR and control + DR remained unchanged following MCT or vehicle injection. This

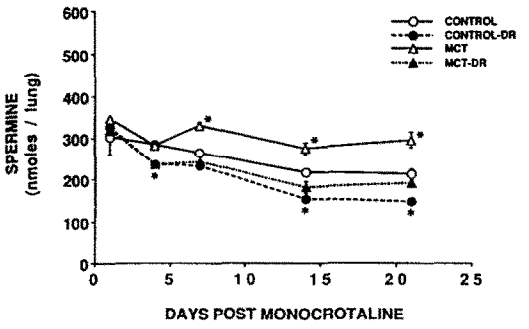

Fig. 8. Changes in the lung spermine content following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). Groups were killed 1, 4, 7, 14, or 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group. Key: (*) significantly different from control at $P < 0.05$.

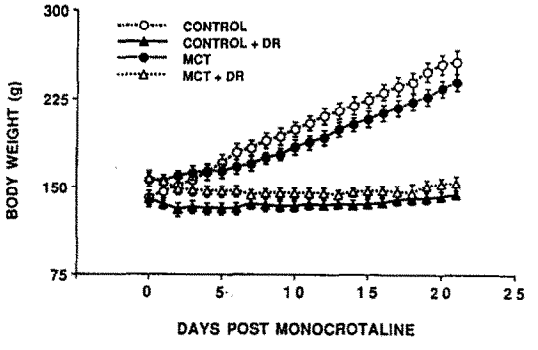

Fig. 9. Changes in body weight following MCT, MCT + DR, and control + DR. Control rats received vehicle (s.c.). All groups were killed 21 days post MCT or vehicle. Values are means ± SEM, N = 5 for each group.

is in contrast to animals in the control or MCT groups where the body weight continued to increase throughout 21 days.

DISCUSSION

The involvement of polyamine biosynthesis in regeneration and growth has been investigated in several organs [34–37]. The polyamines, putrescine, spermidine and spermine, are involved with the regulation of cell growth and differentiation [37–39]. Ornithine decarboxylase is the initial and a rate-limiting enzyme in polyamine biosynthesis [34, 37–39], while *S*-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50) is the rate-limiting enzyme for the conversion of putrescine to spermidine and spermidine to spermine [37–39]. We have found that continuous administration of 2-difluoromethylornithine (DFMO), a specific enzyme activated, irreversible inhibitor of ODC activity [34, 40, 41], prevented the development of MCT-induced PH and RVH [25]. 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 DFMO are unknown.

Although several studies have examined the role of polyamines in chronic lung injury and repair [17–19, 25, 33, 42, 43], there are no reports of the influence of diet on polyamine and DNA synthesis following MCT. The mechanism for protection from MCT-induced PH by DR may involve the prevention of a rise in polyamine and DNA synthesis.

Hayashi *et al.* [8] reported that in MCT-treated diet-reduced animals RVH did not occur; however, some focal swelling of the alveolar walls was noted. Control lungs appeared normal whether the animals were on an *ad lib.* diet or a reduced diet. Dietary reduction also enhanced survival and was associated with a suppression of growth. Our results confirm that total caloric restriction to 45% of usual food intake is associated with a suppression of growth (Fig. 9).

It has been reported that MCT must be metabolized before toxicity is apparent. Ganey *et al.* [44] examined the possibility that DR might protect against MCT-induced toxicity by decreasing the bioactivation of MCT to monocrotaline pyrrole (MCTP). They found that restricting the diet of rats given MCTP prevented RVH when compared with rats given MCTP and placed on an *ad lib.* diet for 14 days. These results support the notion that the inhibition afforded by dietary restriction is due primarily to its action(s) on the events that occur post bioactivation of MCT.

The mechanism for the influence of DR on MCT-induced PH may involve the regulation of ODC activity by the protein content and possibly by the amino acid content of the diet. The activity of ODC increases after a protein meal. Sens *et al.* [45] studied the effect of amino acid mixtures on hepatic and renal ODC activities and polyamine content in postabsorptive and 72-hr fasted rats. They and others [46, 47] have found that fasting decreases ODC activity in liver and kidney, whereas ingestion of a protein, but not a carbohydrate, meal can restore it. Intraperitoneal injection of either 1 g/kg of a synthetic mixture of 17 amino acids or of casein hydrolysate increased renal and hepatic ODC activity 4- and 20-fold, respectively, in fasted rats. In addition, injection of a mixture of glutamate, aspartate, and alanine resulted in increased ODC activity to the same extent as in the 17 amino acid mixture. The content of hepatic and renal putrescine was found to increase in parallel with ODC activity. Increased ODC activity has been correlated with cell growth and proliferation in a variety of systems including partial hepatectomy. It would be important to determine whether a low protein diet might provide protection against MCT-induced PH. Based on the studies cited above, reducing the amino acid content of the diet may be critical in providing protection against MCT-induced PH.

The results of this study are in agreement with the previous findings of Hayashi *et al.* [8] that reduction of total food intake provides protection against

MCT-induced PH. Our study provides new data that are consistent with the hypothesis that DR provides protection against the development of chronic PH in the rat by limiting increases in polyamine biosynthesis. The mechanism by which DR protects against the development of MCT-induced PH is unknown. However, our results suggest that suppression of polyamine biosynthesis by DR may protect against the development of MCT-induced PH in part by preventing increases in DNA synthesis. In a recent study [26] we found that suppression of polyamine biosynthesis by DFMO prevents the increase of DNA synthesis in the lungs of rats exposed to MCT and the development of MCT-induced PH. These data 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. In addition, experiments using ornithine to reverse the effect of DFMO suggest that the development of MCT-induced PH is polyamine dependent and independent of secondary effects upon growth [26]. In the current study we found that DR may protect against the development of MCT-induced PH in part by preventing increases in polyamine biosynthesis that are involved in the regulation of DNA synthesis.

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 [48]. The drug DFMO has been shown to inhibit the synthesis of DNA in a variety of normal and neoplastic cells and tissues [49]. Luk [50] found that DFMO inhibits polyamine and DNA synthesis during liver regeneration following partial hepactectomy. A recent report by Byus and Wu [51] demonstrated that insulin and 12-*O*-tetradecanoylphorbol-β-acetate (TPA) induced DNA synthesis in Reuber H35 hepatoma cells is dependent upon polyamine biosynthesis.

Although we do not know in which cells DNA synthesis is inhibited by DR, we have reported recently that the most sensitive cells to low doses of MCT appear to be endothelial and adventitial cells in the intraacinar vessels of the rat lung [52]. Further studies utilizing DR and specific inhibitors of polyamine metabolism in conjunction with autoradiography will be required to identify which cells are the key lung cells in the proliferative 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.

We conclude that DR to 8 g/day/rat protects against MCT-induced PH and is associated with an inhibition of increased lung polyamine and DNA synthesis that occur in the lung during the development of MCT-induced PH. These results are consistent with a recent report suggesting that increased lung polyamine biosynthesis is required for the development of MCT-induced PH. The data are also consistent with the hypothesis that inhibition of polyamine biosynthesis influences the development of MCT-induced PH in part by regulating DNA synthesis in key lung cells.

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