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Effects of monocrotaline treatment on norepinephrine removal by isolated, perfused rat lungs

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Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid isolated from the leaves and foliage of the *Crotalaria spectabilis* [1]. Administration of MCT to rats results in the development of increased pulmonary arterial pressure, medial thickening in the pulmonary vasculature, and right ventricular hypertrophy [2–4]. In addition, pulmonary capillary endothelial cells show morphological and functional signs of injury. The cells swell and protrude into the vessel lumen and have increased numbers of cytoplasmic organelles and pinocytotic vesicles, as well as enlarged nuclei [5]. These changes are similar to those observed in patients with primary pulmonary hypertension [6], suggesting that MCT-induced pulmonary hypertension in rats might be a useful model for the human disease.

Endothelial cells are the site of a specific, carrier-mediated uptake process for 5-hydroxytryptamine (5-HT) [7, 8]. Monocrotaline treatment decreases the removal of perfused 5-HT by isolated rat lungs [9, 10]. Another biogenic amine, norepinephrine (NE), is also taken into pulmonary endothelial cells by a saturable, carrier-mediated process [7, 11, 12]. Although similar to the 5-HT uptake process, NE uptake seems to occur at pharmacologically different sites [12]. Previous reports of the effects of MCT on NE uptake have presented apparently conflicting observations. One group [9] reported a marked decrease in the NE removal by isolated lungs of rats perfused at 37° after 3 weeks of MCT feeding, but this conclusion was based on a small number of observations (N = 3). Another group [10] using the same treatment regimen detected no changes in NE transport by lungs perfused at room temperature. It would be of interest to know whether MCT does indeed affect NE uptake since human patients with elevated pulmonary vascular resistance have significantly reduced NE removal across the pulmonary circulation [13, 14] and increased plasma NE concentrations [15].

The purpose of this study was to determine whether a single dose of MCT, known to cause pulmonary hypertension, affects NE removal in the isolated, perfused rat lung preparation.

Male, Sprague–Dawley rats (Spartan Farms, Haslett, MI) were used for all experiments. The animals were housed on corn cob derived bedding and allowed free access to food (Wayne Lab-Blox, Continental Brain Co., Chicago, IL) and water. An alternating 12 hr light/dark cycle was maintained. Rats weighing 200–225 g were given a single subcutaneous injection of 105 mg MCT/kg or 0.9% saline. The MCT was dissolved in 0.2 N HCl, neutralized with NaOH, and then brought to volume to provide a working solution of 60 mg MCT/ml. Fourteen days after treatment, NE uptake and metabolism and right ventricular hypertrophy were evaluated.

The isolated, perfused lung preparation has been described in detail previously [3, 9, 16]. Briefly, rats were anesthetized with pentobarbital and treated with 500 units of heparin intravenously. The trachea and the pulmonary artery were cannulated, and the heart was cut away at the level of the atria. The lungs were carefully removed and transferred to a 37° chamber. The perfusion medium was pumped through the lung in a single pass system at a constant flow of 10 ml/min. The perfusion medium consisted of a Krebs–Ringer bicarbonate buffer (pH 7.4) aerated with 95% O_2/5% CO_2 containing 4% bovine serum albumin (Fraction V, Miles Biochemicals, Elkhart, IN) and 4.6 μM calcium disodium edetate.

The airways were filled with 2 ml of room air, and the tracheal cannula was clamped to keep the lungs statically inflated. Inflow perfusion pressure was monitored with a Statham P23ID pressure transducer and recorded on a Grass model 7 Polygraph. After the vasculature was cleared of blood, the perfusion medium was switched to one containing 0.1 μM [^{14}C]norepinephrine (DL-[8-^{14}C]noradrenaline DL-bitartrate, sp. act. 55 mCi/mmole, Amersham, Arlington Heights, IL). Effluent samples were collected 7.5 min after the introduction of [^{14}C]NE. At one end of perfusion, the lungs were removed from the apparatus, blotted, and immediately weighed. Radioactivity in aliquots of perfusion medium was determined directly and after separation on Biorex 70 (pH 6.0) columns. The deaminated metabolites were eluted with water, the unchanged amine was removed by elution with 2% boric acid, and then the O-methylated metabolites were eluted with 0.2 N HCl [17]. Percent removal of NE and percent of perfused NE appearing in the effluent as metabolites were calculated as previously described [3]. Removal of NE is defined as the difference between the concentration of unchanged NE in the inflow perfusion medium and that in the collected effluent. Percent metabolites are expressed as the percentage of perfused NE appearing as metabolites in the effluent perfusate.

Right ventricular hypertrophy was used to confirm the development of pulmonary hypertension. After removing the atria, the right ventricle (RV) was trimmed away, leaving the left ventricle plus septum (LV+S) intact. Each piece was weighed and the ratio RV/(LV+S) was calculated. An increase in RV/(LV+S) in the absence of changes in (LV+S) weight reflects right ventricular hypertrophy [18].

The effects of a single dose of MCT 14 days after treatment are summarized in Table 1. The development of pulmonary hypertension was indicated by right ventricular hypertrophy [an increase in the RV/(LV+S) ratio] and by an increase in the baseline perfusion pressure in the isolated lung preparation. The increased pressure is presumed to reflect an increase in the pulmonary vascular resistance due to medical thickening of the pulmonary blood vessels. The

MCT-treated rats also gained less weight over the course of the experiment and had higher lung weights than control animals, confirming previous results [3].

Removal of NE was decreased 25% by MCT treatment (Table 2). The proportion of NE in the effluent as metabolites was not altered by MCT administration. This result supports the earlier observations of Gillis *et al.* [9] that MCT treatment decreases NE uptake. Huxtable *et al.* [10] reported that MCT did not change NE transport in isolated lungs perfused at room temperature. NE is taken into lung by both active transport and passive diffusion. At 37° the active transport process predominates at the NE concentrations used in these studies. Active transport of biogenic amines is diminished at lower temperatures [7] so that a larger fraction of amine uptake is accounted for by passive diffusion. MCT effects on carrier-mediated transport might have been obscured by perfusing the lungs at 22° instead of 37° in that study [10].

The observed decrease in pulmonary removal likely reflects the endothelial cell damage, evident histologically after treatment of rats with MCT, although other factors may contribute too. Alteration in perfusion variables such as flow, homogeneity of perfusion, and endothelial cell surface area could also be responsible for a decrease in NE uptake [19]. In the case of 5-HT uptake by lungs from MCT-treated rats, lung slices exhibited the same relative decrease in 5-HT uptake as has been described in the isolated perfused lung [20]. This result suggests that at least a large fraction of the decrease in uptake is due to effects on the endothelial cell since lung slices are not perfused and, therefore, are not subject to changes in uptake as a result of perfusion variables. Inasmuch as both NE and 5-HT uptake are subject to similar dependence on perfusion variables, the decreased NE uptake observed in these experiments may be largely a function of endothelial cell injury.

Available evidence suggests that the effects of MCT on the endothelial cell are somewhat non-specific. 5-HT and NE transport processes are both depressed [3, 9, 10], the activity of a luminal surface enzyme, angiotensin converting enzyme, is altered [4], and preliminary work suggests that prostacyclin synthesis in the lung is increased [21]. These functions all may affect concentrations of vasoactive agents in the pulmonary vasculature. It is possible that these functional alterations play a role in the development and/or maintenance of increased pulmonary arterial pressures after MCT administration. Experimental support for such speculation is not yet available.

In summary, a single dose of MCT (105 mg/kg, s.c.) resulted in right ventricular hypertrophy and a 25% decrease in NE removal by isolated lung preparations. The percentage of perfused NE in the effluent perfusion medium appearing as metabolites was not altered by MCT treatment. These results clarify previous apparently disparate observations of the effect of MCT on NE uptake and provide further evidence of the similarity between MCT-induced pulmonary hypertension in the rat and human primary pulmonary hypertension.

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Table 1. Monocrotaline effects

Treatment*	Body wt gain (g)	Wet lung wt (g)	RV/(LV+S)	$\frac{RV}{\text{Body wt}} \times 100$	Initial perfusion pressure (mm Hg)
Saline	99 ± 4	1.65 ± 0.04	0.294 ± 0.008	0.072 ± 0.001	5 ± 1
MCT	18 ± 9†	2.59 ± 0.18†	0.338 ± 0.009†	0.111 ± 0.014†	8 ± 1†

*Rats were treated with 105 mg MCT/kg or an equivalent volume of 0.9% saline (s.c) 14 days prior to use. Body weight gain is the difference between body weight at 14 days and body weight at the time of treatment (day 0). Values are reported as mean ± S.E.M. N = 13 in each group.

†Significantly different from saline group (Student's *t*-test, $P < 0.05$).

Table 2. Effect of MCT treatment of rats on norepinephrine removal and metabolism in the isolated, perfused lung

Treatment*	NE removal (%)	Deaminated metabolites (%)	O-Methylated metabolites (%)
Saline	20.6 ± 1.7	9.9 ± 1.0	5.3 ± 0.9
MCT	15.4 ± 1.3†	8.3 ± 0.9	3.8 ± 1.0

*Rats were treated with 105 mg MCT/kg or an equivalent volume of 0.9% saline (s.c.) 14 days before perfusion. Norepinephrine (NE) removal and metabolism were determined as described in the text. Values are reported as mean ± S.E.M. N = 13 in each group.

†Significantly different from saline group (Student's *t*-test, $P < 0.05$).

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Hypoxanthine-guanine phosphoribosyltransferase-independent toxicity of azathioprine in human lymphoblasts

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6-Mercaptopurine (6-MP*) and its imidazole derivative, azathioprine (AZ), are clinically useful immunosuppressive agents. AZ in particular has gained widespread use and has been cited as "the most widely used cytotoxic immunosuppressive agent in clinical medicine" [1]. The biochemical and metabolic effects of these thiopurines have been reviewed [1–4]. However, the precise mechanism of their immunosuppressive activity is not clearly understood [1,2].

The cytotoxicity of 6-MP is dependent upon its phosphoribosylation to thioinosinic acid (TIMP) which is catalyzed by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT, EC 2.4.2.8). Cell lines which are resistant to 6-MP have been shown to lack HPRT activity [5], and neither 6-MP nor AZ has activity in HPRT-deficient patients [6]. TIMP is an effective inhibitor of purine nucleotide biosynthesis and interconversion (see Fig. 1). It is a competitive inhibitor of adenylsuccinate synthetase, adenylsuccinate lyase, and IMP dehydrogenase, and it is a pseudo feedback inhibitor of glutamine-phosphoribosylpyrophosphate amidotransferase, the first enzyme unique to the *de novo* purine nucleotide biosynthetic pathway [3,4]. Other effects of TIMP include incorporation into nucleic acid, the inhibition of coenzyme formation and function, and the inhibition of protein synthesis [3,4].

Many of the metabolic effects of AZ are dependent upon its cleavage to 6-MP and the subsequent phosporibosylation to TIMP. However, there has been increasing evidence which indicates that AZ has metabolic effects which may account for its distinct and more widespread clinical applicability as an immunosuppressant as compared to 6-MP [1–4,7,8]. These effects might be attributable to biologically active metabolites unique to AZ or could possibly be due to the reaction of the methylnitroimidazole moiety with active metabolites or with potentially reactive groups on cellular proteins [2].

In this study, utilizing HPRT$^+$ and HPRT-deficient human lymphoblastoid cell lines, we have found evidence which suggests that the cytotoxicity of AZ in cell culture, unlike that of 6-MP, is, in part, independent of HPRT activity.

Materials and methods

Chemicals. Azathioprine, 6-mercaptopurine, glutamine and the purine nucleosides and bases used were purchased from the Sigma Chemical Co. Heat-inactivated horse serum and RPMI 1640 medium were purchased from the Grand Island Biological Co. RPMI 1640 contains glutathione (1 mg/l) as one of its components.

Cell lines. Human lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 2 mM glutamine. The MGL-8 cell line (HPRT$^+$) was derived from a normal individual and was a gift from J. Epstein, Johns Hopkins University. The GM-130 cell line, derived from a normal individual, and the GM-467 (HPRT-deficient, mutagenized) cell line were obtained from the Human Genetics Mutant Cell Repository. The HPRT-deficient cell line HD was derived from a patient presenting with the Lesch–Nyhan Syndrome by Epstein–Barr virus transformation by the method described previously [9]. The MOLT-4 cell line (HPRT$^+$), originally derived from a patient presenting with the Lesch–Nyhan Syndrome by Epstein–Barr virus transformation by the method described previously [9]. The MOLT-4 cell line (HPRT$^+$), originally derived from a patient with acute

* Abbreviations: 6-MP, 6-mercaptopurine; AZ, azathioprine; TIMP, thioinosinic acid; and HPRT, hypoxanthine-guanine phosphoribosylatransferase.