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SHORT COMMUNICATION

Effects of Monocrotaline, a Pyrrolizidine Alkaloid, on Glutathione Metabolism in the Rat

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ABSTRACT. Monocrotaline (MONO), a pyrrolizidine alkaloid, causes veno-occlusive disease of the liver, pulmonary arterial hypertension, and right ventricular hypertrophy. Toxicity is due to the hepatic formation of a pyrrolic metabolite that can be detoxified by conjugation with glutathione (GSH). We have shown that the GSH content of the liver affects the quantity of the pyrrolic metabolite that is released from the liver. We have now examined whether MONO, in turn, affects GSH metabolism. Twenty-four hours after administration of MONO to rats (65 mg/kg, i.p.), the highest concentration of bound pyrrolic metabolites was found in the liver, followed by the lung and kidney. Heart and brain contained lower concentrations of these metabolites. Significantly higher levels of GSH were found in liver and lungs of MONO-treated rats than in saline-injected control animals. In the liver, activities of the following enzymes were elevated: γ-glutamylcysteine synthetase, GSH synthetase, γ-glutamyl transpeptidase, dipeptidase, and microsomal GSH transferase. The same changes were seen in the lung. In the heart, γ-glutamyl transpeptidase activity was decreased markedly, and cytosolic GSH transferase activity was elevated. In the kidney, the activities of GSH synthetase, γ-glutamyl transpeptidase, and cytosolic GSH transferase were increased. Our results establish a mutual interaction of MONO and sulfur metabolism. It appears that an early metabolic action of MONO is to modify sulfur amino acid metabolism, diverting cysteine metabolism from oxidation to taurine towards synthesis of GSH. BIOCHEM PHARMAC 51;3:000–000, 1996.

KEY WORDS. γ-glutamylcysteine synthetase; γ-glutamyl transpeptidase; pyrrolic metabolites; GSH *S*-transferases

PAs† are widespread plant toxins, responsible worldwide for numerous public health and economic problems [1]. Among the major toxic actions of PAs are liver cancer, hepatomegaly, and veno-occlusive disease of the liver. In addition, the toxicity of certain PAs can also be expressed in extrahepatic organs, including the heart, lung, kidney, and central nervous system [1–3].

MONO is a pneumotoxic PA, causing pulmonary arterial hypertension and right ventricular hypertrophy after single doses of 60–105 mg/kg [4, 5]. Toxicity is the result of hepatic bioactivation to the toxic, alkylating pyrrole, dehydromonocrotaline [6, 7]. Dehydromonocrotaline can alkylate cell macromolecules in the liver, with such alkylation probably representing the biochemical basis of its toxicity [8, 9]. It can also be released into the circulation to bind covalently to macromolecules in extrahepatic organs [8–10]. The amount of dehydromonocrotaline available for these presumably intoxicating pathways is affected markedly by the GSH content of the liver [11]. GSH conjugates with dehydromonocrotaline to form GSDHP, a compound of much lower toxicity that is released in high concentration into the bile [8]. Sulfur amino acids, such as methionine and Cys, that elevate hepatic GSH content also protect against PA toxicity [12–14].

We have established in the isolated, perfused liver a correlation between hepatic GSH concentration and bile GSDHP formation, indicating that the size of the hepatic GSH pool influences the metabolism and resulting toxicity of MONO [11]. The influence of PAs on sulfur metabolism is much less clearly understood, and has, indeed, been largely ignored. We have shown recently that *in vivo* exposure to PAs leads to an increase in hepatic GSH concentration via the stimulation of overall GSH synthesis [10]. We now report an examination of the mechanism of this increase.

MATERIALS AND METHODS

Chemicals

GSH, L-Cys, L-glutamic acid (Glu), DTT, γ-GluCys, CysGly, GlyGly, *N*-ethylmorpholine, and the kit for protein determination were purchased from the Sigma Chemical Co. (St. Louis, MO). MB (Thiolyte Reagent) was purchased from Cal-

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† *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; CysGly, cysteinylglycine; DTT, DL-dithiothreitol; γ-GluCys, γ-glutamylcysteine; GlyGly, glycylglycine; GSDHP, 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5*H*-pyrrolizine; GSH, glutathione; MB, monobromobimane; MONO, monocrotaline; and PA, pyrrolizidine alkaloid.

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biochem (San Diego, CA). MONO was isolated from the seeds of *Crotalaria spectabilis* [15]. Ehrlich reagent (0.5 g *p*-dimethylaminobenzaldehyde in 25 mL absolute ethanol acidified with 0.35 mL of 70% perchloric acid) was prepared daily.

Treatment Protocol

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from the University of Arizona Division of Animal Resources and housed 2–3 to a cage in a room maintaining 12-hr cycles of light and dark. After 3 days, they were divided into two groups of 5 animals. Rats in the MONO group were administered MONO (65 mg/kg body weight, i.p.), and rats in the control group 0.85% NaCl (the same volume as for the MONO group, i.p.). They were allowed food and tap water *ad lib*. Twenty-four hours after being injected, all rats were killed by decapitation. To avoid diurnal variation in enzyme activities, all rats were killed between 9:00 and 10:00 a.m.

Tissue Cytosol and Microsomal Preparation

Tissues were removed and washed twice in saline at 4°. Each tissue was minced and homogenized in 4 vol. of 0.25 M sucrose containing 20 mM Tris–HCl and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 3,000 *g* for 10 min at 4° and then the supernatant was centrifuged at 10,000 *g* for 20 min at 4°. Finally, the supernatant was centrifuged at 105,000 *g* for 60 min at 4°. The supernatant was used to determine GSH concentration and the activities of γ-GluCys synthetase, GSH synthetase and cytosolic GSH *S*-transferase, and the microsomal pellet was used to determine the activities of dipeptidase, γ-glutamyl transpeptidase and microsomal GSH *S*-transferase.

Enzyme Assays

Enzyme-activities were determined by HPLC analysis of the MB derivatives of products. Enzyme activities were determined by incubation of protein at 37° with substrates, coenzymes, and metal ions. Reactions were terminated by adding 100 μL of incubate to 50 μL of 5% sulfosalicylic acid and centrifuging the mixture for 3 min to precipitate protein. The supernatant was derivatized with MB as previously described [16] and injected onto HPLC. Peaks were identified by comparison with the retention times of authentic standards and quantified by comparison with standard curves.

Cytosolic γ-GlyCys Synthetase and GSH synthetase

Cytosolic γ-GluCys synthetase activity was determined by following formation of γ-GluCys from Cys and glycine [17]. Assay of γ-GluCys synthetase activity was performed in 1.5-mL microfuge tubes containing 10 mM Glu, 5 mM Cys, 10 mM ATP, 0.1 M Tris–HCl (pH 8.2), 0.15 M KCl, 2 mM EDTA and 20 mM $MgCl_2$. After preincubation for 3 min at 37°, cytosolic protein (about 1 mg) was added to initiate the enzyme reaction. The total volume was 1.0 mL, and the incubation time was 20 min. The characterization of the enzyme under these conditions has been reported [17].

The assay of GSH synthetase activity was the same as that of γ-GluCys synthetase, except that Glu and Cys were replaced by 10 mM glycine and 5 mM γ-GluCys. The GSH content in the incubation at time 0 was subtracted from the final GSH content to calculate the newly synthesized GSH.

Microsomal γ-Glutamyl Transpeptidase and Dipeptidase

Microsomal γ-glutamyl transpeptidase was assayed based on the release of CysGly from GSH [18]. The standard assay solution (1.0 mL) contained 0.1 mM Tris–HCl buffer (pH 8.0), 75 mM NaCl, 20 mM GlyGly, 5 mM GSH, 50 μM DTT and microsomal protein (0.5 mg). The solution was equilibrated at 37°. Reaction was initiated by addition of protein, and the solution was incubated for 20 min. To correct for hydrolytic release of CysGly from GSH by γ-glutamyl transpeptidase, a blank tube lacking the substrate GlyGly was prepared at the same time.

Microsomal dipeptidase was assayed based on Cys release from CysGly. The incubation system contained 2 mM CysGly, 75 mM NaCl, and 0.1 M Tris–HCl (pH 8.0). The final volume of the reaction mixture was 1.0 mL. The solution was brought to 37° and incubated for 20 min. To correct for hydrolytic release of Cys from CysGly, a blank tube lacking microsomal protein was included.

HPLC Procedure

HPLC was performed as previously described [16]. Retention times for Cys, CysGly, γ-GluCys and GSH were 7.12, 8.34, 9.93 and 11.40 min, respectively.

Freshly prepared solutions of Cys, CysGly, γ-GluCys or GSH were added to microfuge tubes containing 20 μL of 50 mM *N*-ethylmorpholine (pH 8.7) and 10 μL of 50 mM MB in acetonitrile. The mixtures were incubated in the dark at room temperature for at least 15 min and then diluted with water. Twenty microliters of the diluted mixture was injected onto the HPLC. The areas of the eluted peaks were integrated to derive standard curves. Aliquots from enzyme incubations (100 μL) were put into microfuge tubes containing 5% sulfosalicylic acid (50 μL) and centrifuged for 3 min to precipitate protein. Supernatants (100 μL) were taken and derivatized as described above.

Cytosolic and Microsomal GSH S-Transferase

GSH *S*-transferase activity was assayed with CDNB according to Habig *et al.* [19]. In brief, incubations contained 1 mM GSH, 0.13 M phosphate buffer (pH 7.0) and 50 μg protein. The total incubation volume was 1 mL. Mixtures were preincubated at 37° for 3 min. The reaction was started by adding 1 mM CDNB (final concentration), and incubation was continued for another 5 min. Under these conditions, product formation was directly proportional to incubation time and protein content. The reaction was stopped by addition of 5%

sulfosalicylic acid, followed by centrifugation to precipitate protein. Appropriate controls were included for determination of spontaneous (nonenzymatic) conjugation.

Tissue-Bound Pyrrole Determination

Tissue-bound pyrrole content was determined as described earlier [9, 20].

Protein Assay

Protein assay was performed according to Lowry *et al.* [21], using bovine serum albumin as standard.

Statistics

Statistical comparisons were performed using Student's unpaired *t*-test.

RESULTS

Male Sprague–Dawley rats (200–250 g) were injected i.p. with either MONO (65 mg/kg) or saline as control, and the animals were killed 24 hr later. The dose of MONO represents 60% of the LD_{50} in adult male rats (deaths within 4–7 days) [22]. Over the 24-hr period, this dose did not alter significantly body weight, liver weight, or the liver to body weight ratio, compared with the control rats.

At 24 hr, bound pyrrolic metabolites of MONO were detected in all tissues examined in the MONO-treated animals (Fig. 1). The highest concentration of pyrrolic metabolites was found in the liver. Heart and brain contained the lowest concentrations of such metabolites.

Cytosolic GSH concentrations of liver and lung in MONO-treated animals were significantly higher than in control rats. No differences were found in heart or kidney (Table 1).

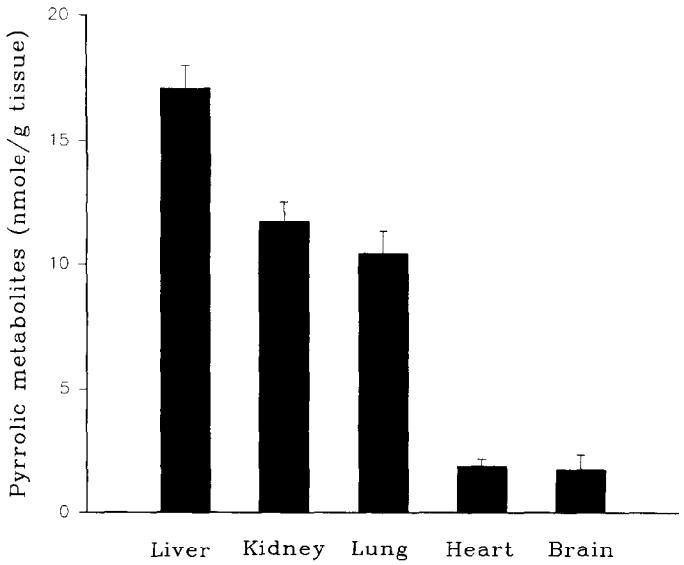

FIG. 1. Tissue-bound pyrrole in rats treated with MONO (65 mg/kg body weight) for 24 hr. Data are means ± SD for 5 samples per point.

The biosynthesis of GSH is mediated by γ-GluCys synthetase and GSH synthetase. In control rats, the highest activity of γ-GluCys synthetase was found in the kidney. The levels of GSH synthetase activity fell in the order of kidney, liver, lung, and heart (Table 1). The activities of both enzymes were increased significantly in liver and lung of the MONO-treated rats. Significantly higher activity of renal GSH synthetase was also seen in the MONO-treated than in the control rats (Table 1).

The catabolism of GSH is initiated by γ-glutamyl transpeptidase, located at the cell membrane. This enzyme cleaves GSH to release CysGly. In MONO-treated animals, γ-glutamyl transpeptidase activity was significantly higher in liver, lung and kidney, but markedly lower in heart, compared with the control group (Table 1). The product, CysGly, is further metabolized by membrane-bound dipeptidase. Rats treated with MONO showed 90% higher dipeptidase activity in both liver and lung compared with the control group. However, no difference was found in dipeptidase activity in heart and kidney between treated and control groups (Table 1).

GSH *S*-transferases comprise a family of enzymes occurring both in the cytosol and microsomal fraction of many tissues. In MONO-treated rats, cytosolic GSH *S*-transferase activity was elevated significantly in heart and kidney (Table 1). An opposite pattern was found for microsomal transferase, this being elevated only in liver and lung in MONO-exposed rats (Table 1).

DISCUSSION

MONO is a toxic PA that is widespread in *Crotalaria* species. It produces pathological lesions in liver, lung, kidney and heart. Toxicity is caused by bioactivation of MONO in the liver to the reactive, alkylating pyrrole, dehydromonocrotaline. Such a mechanism was suggested in 1968 by Mattocks [7], and recently verified with the isolation of dehydromonocrotaline from incubations of rat liver microsomes [6] and the demonstration that dehydromonocrotaline is released from isolated livers perfused with MONO [8]. Dehydromonocrotaline has the ability to alkylate cell macromolecules. Synthetic dehydromonocrotaline reproduces the toxicity of MONO [23–25]. The distribution of tissue binding of pyrrolic metabolites of MONO (Fig. 1) is in keeping with its high hepatic and pulmonary toxicity, and the renal excretion of the parent PA and its metabolites.

Single doses of MONO in the range 60–105 mg/kg produce pulmonary arterial hypertension and right ventricular hypertrophy in rats over the succeeding 2 weeks [4, 5]. We therefore chose to examine the effects of 65 mg/kg on GSH metabolism.

In the isolated liver, dehydromonocrotaline readily conjugates with GSH to form the less toxic secondary metabolite, GSDHP [9]. This is excreted in high concentrations into the bile. MONO (0.5 mM) also induces a 30-fold increase in the biliary excretion of GSH in the isolated, perfused liver [16]. As a result, GSH levels fall in the MONO-perfused, isolated, rat

TABLE 1. Effect of monocrotaline on cytosolic GSH concentration and the activities of enzymes involved in GSH metabolism

			Enzyme activity (nmol/mg protein/min)					
Tissue	Treatment	GSH (nmol/mg protein)	γ-Glutamylcysteine synthetase	GSH synthetase	γ-Glutamyl transpeptidase	Dipeptidase	GSH S-transferase (cytosolic)	GSH-S-transferase (microsomal)
Liver	Control	97.0 ± 11.6	7.0 ± 0.8	4.63 ± 0.38	0.90 ± 0.08	0.53 ± 0.05	388 ± 36	194 ± 25
	MONO*	162 ± 18†	9.3 ± 1.3†	5.86 ± 0.51†	1.08 ± 0.09†	0.93 ± 0.12†	491 ± 107	259 ± 22†
Heart	Control	57.0 ± 3.8	1.4 ± 0.2	1.02 ± 0.12	26.7 ± 2.0	0.62 ± 0.08	36.4 ± 6.1	ND‡
	MONO	56.4 ± 6.2	1.6 ± 0.2	1.06 ± 0.09	4.19 ± 0.42†	0.51 ± 0.06	67.3 ± 4.4†	ND
Lung	Control	42.5 ± 8.8	0.9 ± 0.0	1.26 ± 0.09	12.2 ± 1.6	1.45 ± 0.13	54.0 ± 8.4	18.1 ± 3.1
	MONO	65.1 ± 5.4†	1.3 ± 0.3†	1.45 ± 0.14†	23.0 ± 2.8†	2.74 ± 0.40†	63.3 ± 4.2	24.8 ± 1.2†
Kidney	Control	60.4 ± 2.2	132 ± 8	39.2 ± 3.6	8,360 ± 680	69.9 ± 3.8	104 ± 12	42.3 ± 2.6
	MONO	53.7 ± 7.8	138 ± 10	46.4 ± 0.8†	10,230 ± 820†	70.5 ± 3.0	146 ± 13†	45.9 ± 4.5

* Rats were injected i.p. with monocrotaline (65 mg/kg) 24 hr before being killed. Data are means ± SD for 5 animals per group.
† $P < 0.05$ relative to the control group.
‡ Not detected.

liver [26]. In view of these observations, it was surprising to find that 24 hr following *in vivo* treatment of rats with the related PAs, MONO or trichodesmine, GSH levels were increased in the liver [10]. In the present study, we also found an increased GSH content in lung and liver (Table 1).

Hepatic GSH levels are determined by a balance of synthesis, degradation, conjugation, and release. An increased level in the face of increased conjugation and release implies an increase in the net rate of synthesis. We have examined whether this is so following exposure to MONO by an application of novel analytic procedures to a study of the enzymes involved in GSH turnover [17].

GSH is biosynthesized by the consecutive actions of γ-GluCys synthetase and GSH synthetase. The breakdown of GSH is catalyzed by membrane-bound γ-glutamyl transpeptidase, with the accompanying formation of CysGly. The latter dipeptide can be further metabolized to free Cys under the action of microsomal dipeptidase. The increased GSH concentration we observed after MONO exposure appears to be largely the consequence of increased synthesis, higher activities of both γ-GluCys synthetase and GSH synthetase being found (Table 1). GSH catabolism is increased in the liver, lung and kidney following administration of MONO (Table 1). The higher GSH concentration observed in the MONO-treated animals, therefore, is associated with increased turnover of GSH, both its synthesis and degradation increasing. In MONO-treated rats, hepatic microsomal GSH *S*-transferase activity also was increased significantly (Table 1).

Although no change in GSH level was seen in the heart, MONO still modified the metabolism of this thiol, in that GSH transferase activity was elevated (Table 1), while γ-glutamyl transpeptidase activity was inhibited markedly (Table 1).

Our results establish a mutual interaction of PAs and sulfur metabolism. Thiol agents, such as GSH, detoxify dehydromonocrotaline by conjugation to the less toxic metabolite, GSDHP. However, PAs such as MONO in turn modify sulfur amino acid metabolism, diverting Cys metabolism from oxidation to taurine [27] towards GSH synthesis. This carries implications for the public health risks of PAs, in that toxicity at a given level of exposure may be conditionally dependent on the dietary sulfur amino acid status, and hence on the general state of protein nutrition.

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