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In Vivo Detoxification of Cyanide by Cystathionase γ-Lyase

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ABSTRACT. The results of several *in vitro* studies have suggested that the enzyme cystathionase γ-lyase (EC 4.4.1.1) may function in the endogenous detoxification of cyanide; however, this possibility has not been investigated *in vivo*. If cystathionase γ-lyase is involved in the endogenous detoxification of cyanide, it logically follows that inhibiting cystathionase γ-lyase should increase the toxicity of cyanide. To test this hypothesis, the activity of cystathionase γ-lyase was inhibited with a suicide inhibitor, 2-amino-4-pentynoic acid (propargylglycine). The activity of liver cystathionase γ-lyase activity was decreased 96.8% by administration of propargylglycine, indicating that the propargylglycine treatment was effective. The propargylglycine treatment did not alter the activity of thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1) or 3-mercaptopyruvate:cyanide sulfurtransferase (EC 2.8.1.2), two other enzymes that have been proposed to be involved in the detoxification of cyanide. The LD_{50} of cyanide in rats treated with propargylglycine was 5.14 ± 0.029 mg NaCN/kg, which was significantly ($P < 0.05$) lower than the 5.98 ± 0.008 mg NaCN/kg LD_{50} of cyanide determined in control rats. The results of these studies suggest that cystathionase γ-lyase may participate in the detoxification of cyanide *in vivo*. BIOCHEM PHARMACOL 52;6:941–944, 1996.

KEY WORDS. cystathionase γ-lyase; rhodanese; 3-mercaptopyruvate sulfurtransferase; cyanide detoxification; thiocyanate

The oxidation of CN‡ to SCN is the primary *in vivo* biochemical pathway for CN detoxification [1]. Thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1; rhodanese) and 3-mercaptopyruvate:cyanide sulfurtransferase (EC 2.8.1.2; MST) are thought to be the major enzymes involved in the detoxification of CN. Rhodanese transfers sulfur donated from a sulfane-sulfur source, such as thiosulfate, to CN [2], whereas MST catalyzes the transfer of sulfur from 3-mercaptopyruvate to CN [3], both forming SCN.

There is also evidence that a third enzyme, cystathionase γ-lyase (EC 4.4.1.1; cystathionase), may participate in the endogenous detoxification of CN. In contrast to the direct conjugation reactions catalyzed by rhodanese and MST, the reactions catalyzed by cystathionase that are hypothesized to be involved in CN detoxification are not direct conjugation reactions. Using a coupled *in vitro* enzyme system between cystathionase and rhodanese, it was demonstrated that cystathionase greatly enhanced the transsulfuration of CN to SCN, and it was suggested that a product of the cystathionase reaction, bis(2-amino-2-carboxyethyl)trisulfide (thiocystine), is serving as a sulfur donor substrate for rhodanese [4]. This hypothesis was strengthened by the observations that purified thiocystine enhanced the rhodanese-catalyzed transsulfuration of CN to SCN 7-fold in comparison to thiosulfate [4], and an *in vivo* study determined that thiocystine protected rats from 2–3 LD_{50} of CN [5]. Another product of the cystathionase reaction, 3-(thiosulfeno)-alanine (thiocysteine), may represent an additional link between cystathionase and CN detoxification. Thiocysteine has been demonstrated to transsulfurate hypotaurine (2-aminoethanesulfinic acid) to thiotaurine (2-aminoethanethiosulfonic acid [6]. *In vitro* studies have determined that thiotaurine is an excellent substrate for rhodanese [7], and *in vivo* thiotaurine is an effective antidote for CN [8].

These studies have provided *in vitro* evidence for the possibility that cystathionase may participate in the endogenous CN detoxification system. Thus, it would be expected that, if cystathionase is involved in the endogenous detoxification of CN, inhibiting cystathionase should enhance the toxicity of CN. The purpose of this study was to examine whether the enzyme cystathionase is responsible, in part, for the *in vivo* detoxification of CN.

DISCLAIMER: The opinions or assertions contained in this paper are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or Department of Defense. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, National Research Council.

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‡ *Abbreviations:* CN, cyanide; SCN, thiocyanate; and MST, 3-mercaptopyruvate:cyanide sulfurtransferase.

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MATERIALS AND METHODS

Chemicals

Sodium thiosulfate, 2-amino-2-methyl-1,3-propanediol, pyridoxal phosphate, 2-mercaptoethanol, and 2-amino-4-pentynoic acid (propargylglycine) were purchased from the Sigma Chemical Co. (St. Louis, MO). DL-Homoserine was purchased from the Fluka Chemical Corp. (Ronkonkoma, NY). KCN was purchased from Mallinckrodt (Chesterfield, MO) and sodium 3-mercaptopyruvate from the United States Biochemical Corp. (Cleveland, OH). All other chemicals were analytical grade reagents.

Animals

A total of twenty-four male Sprague–Dawley rats (176–200 g) were used in these studies. All animals were maintained under an AAALAC accredited animal care and use program. Animals were quarantined and observed for evidence of disease for 5 days prior to use. Animals were housed in polycarbonate cages, with one animal/cage, on corn cob bedding (Bed O'Cobs, Andersons COB Division, Maumee, OH) with complete cage changes twice weekly. Commercial Certified Rat Chow and tap water were provided *ad lib.* Animal holding rooms were maintained at 21 ± 2° with 50 ± 10% relative humidity with at least 10 complete air changes per hour of 100% conditioned fresh air. A 12-hr light/dark, full spectrum lighting cycle with no twilight was maintained in the animal holding area.

LD_{50} *Studies*

The LD_{50} studies were designed and conducted using the up-and-down method [9] to minimize animal usage, and a total of twelve rats were used in these studies. Six rats were administered propargylglycine (250 μmol/kg) by i.p. injection using 145 mM NaCl as the vehicle at 24-hr intervals on 3 consecutive days. This dose of propargylglycine was chosen because it had been shown previously to decrease significantly cystathionase activity in rats [10]. Six rats which served as controls received injections of 145 mM NaCl (1 mL/kg) at 24-hr intervals on 3 consecutive days. Injections of NaCN were made exactly 24 hr after the third administration of propargylglycine or 145 mM NaCl. Using the LD_{50} value of 5.87 mg NaCN/kg (i.p. injection route) previously reported for rats [11], doses of NaCN at 0.075 log intervals from this LD_{50} value were used. NaCl (145 mM) was used as the vehicle for CN injections, and these injections were made i.p. Any animals that survived the CN challenge were euthanatized with 100% CO_2 24 hr after completion of the experiment.

Liver Isolation and Homogenization

Twelve rats were divided into two groups of six rats each. One group of rats received propargylglycine injections, and the other group of six rats received injections of 145 mM NaCl (1 mL/kg) exactly as described for the LD_{50} studies. Twenty-four hours after the third administration of propargylglycine or 145 mM NaCl, the rats were euthanatized with CO_2 followed by bilateral thoracotomy. Livers were removed immediately *en bloc*, flushed with 145 mM NaCl, frozen in dry ice, placed in plastic vials, and stored at −80°. Fifty percent (w/v) liver homogenates were prepared by homogenizing liver samples in 0.1 M potassium phosphate buffer (pH 7.4) with a Polytron homogenizer for 3 min at 20% power, followed by 3 min at 80% power. All homogenizations were done on ice. Insoluble material was removed by centrifugation (3000 *g* for 5 min at 4°), and protein was determined using a dye binding assay [12]. After centrifugation, liver homogenates were stored at −80°.

Cystathionase Assay

Cystathionase activity was measured using liver homogenates prepared as described above using a modification of a previously described assay [13]; the measurements were conducted under yellow light due to the light sensitivity of pyridoxal phosphate. The assay contained 100 mM potassium phosphate buffer (pH 7.5), 16 mM DL-homoserine, 50 μM pyridoxal phosphate, 7.5 mM 2-mercaptoethanol, and 100 μL of liver homogenate (4.7 to 6.7 mg protein) in a total volume of 500 μL. All of the components of the assay were preincubated at 37° for 2 min except for DL-homoserine, which was added to initiate the assay. The reaction was allowed to proceed for 30 min and was terminated by the addition of 2.0 mL of 10% trichloroacetic acid. Precipitated protein was pelleted by centrifugation, and the supernatant was removed for the determination of α-ketobutyric acid. α-Ketobutyric acid was determined by a colormetric assay as previously described [13] with the following modifications: 0.5 mL of the assay supernatant was used, and thus all other reagents were added at half their reported volumes.

MST Assay

Liver homogenate was diluted 1:10 (v/v) with 200 mM phosphate buffer (pH 7.4), and MST activity was measured using a previously described assay [3] with modifications. The MST assay contained 220 mM 2-amino-2-methyl-1,3-propanediol (pH 9.5), 15 mM sodium 3-mercaptopyruvate, 12.5 mM KCN, and 20 μL diluted liver homogenate (0.8 to 0.14 mg protein) in a total volume of 500 μL. All of the components of the assay were preincubated at 30° for 2 min except for KCN, and the assay was initiated by the addition of KCN. The reaction was allowed to proceed for 15 min and was terminated by the addition of 0.25 mL of 38% formaldehyde. Enzymatic formation of SCN was corrected for non-enzymatic reactions between 3-mercaptopyruvate and CN by subtracting the mean absorbance of duplicate reaction tubes incubated under identical conditions with enzyme omitted. SCN was determined as a ferric ion complex in a colorimetric assay at 460 nm as previously described [14].

Rhodanese Assay

Liver homogenate was diluted 1:10 (v/v) with 200 mM phosphate buffer (pH 7.4), and rhodanese activity was measured using a previously described assay [2] with modifications. The assay consisted of 40 mM phosphate buffer (pH 8.6), 40 mM sodium thiosulfate, 40 mM KCN, and 10 μL diluted liver homogenate (0.04 to 0.07 mg protein) in a volume of 500 μL. After preincubation at 30° for 2 min, the reaction was initiated by the addition of KCN and allowed to proceed for 15 min. The reaction was stopped with the addition of 0.25 mL of 38% formaldehyde, and SCN production was determined as previously described. Enzymatic formation of SCN was corrected for non-enzymatic reactions between thiosulfate and CN by subtracting the mean absorbance of duplicate reaction tubes incubated under identical conditions with enzyme omitted.

Statistical Analysis

The LD_{50} values (±SEM) of CN were calculated by probit analysis [15], and protective ratios [16] were determined as previously described. Significant differences between enzyme activities in control versus propargylglycine-treated animals were determined by a two-tailed *t*-test.

RESULTS AND DISCUSSION

Propargylglycine was selected as the cystathionase inhibitor for use in this study because it was predicted *a priori* that it would effectively inhibit cystathionase *in vivo* but would not affect the activity of rhodanese or MST. This prediction was based on the specificity and mechanism of propargylglycine inhibition which had been determined previously [17], and the observation that propargylglycine is a highly effective inhibitor of cystathionase *in vivo* [10]. The results of the enzyme assays on the liver homogenates are presented in Table 1. Propargylglycine reduced the activity of cystathionase 96.8%, which represents a significant decrease ($P < 0.05$) in comparison to the control. This percent decrease was similar to that previously reported with the same dose of propargylglycine in rats [10]. Propargylglycine did not have a significant effect ($P > 0.05$) on rhodanese or MST activities in comparison to control. Thus, any effect on the toxicity of CN in propargylglycine-treated rats cannot be from an inhibition of rhodanese or MST, suggesting that it is due to the inhibition of cystathionase.

The LD_{50} of CN for the control animals was 5.98 ± 0.008 mg NaCN/kg, which is in close agreement with a previously reported value [11]. Rats treated with propargylglycine had a CN LD_{50} of 5.14 ± 0.029 mg NaCN/kg, which represents a significant decrease ($P < 0.05$) in comparison to control rats. The protective ratio was determined to be 0.85 (95% confidence intervals = 0.75, 0.98). Because the 95% confidence interval of the protective ratio does not include 1.0, this protective ratio (0.85) represents a significant difference. Although statistically significant, the approximately 14% decrease in the LD_{50} of CN may not appear to be biologically significant. However, the dose–response curve of CN is quite steep [18], and thus a small shift in the LD_{50} can reflect a significant biological difference in the toxicity of CN.

Comparison of the total estimated sulfane-sulfur available in humans for cyanide detoxification has been compared to the lethal dose of CN [19] and suggests that insufficient sulfane-sulfur exists to detoxify a lethal dose of CN under steady-state conditions. Thus, therapies for CN intoxication often include the administration of supplemental sulfane-sulfur compounds, like sodium thiosulfate [20]. Cystine has been known to have protective effects against lethal doses of CN when injected prior to CN [21]. A previous study suggested that this antidotal action of cystine may be due to the formation of 2-imino-4-thiazolidine via a non-enzymatic reaction between cystine and CN [22], but whether this reaction is quantitatively significant *in vivo* remains to be determined [23].

Cystine is also a substrate for cystathionase [24], but because tissue cystine levels are low under normal physiological conditions [25], cystathionase activity may not be at its optimum rate. However, the results of numerous *in vitro* assays have led to the suggestion that the products of cystathionase-catalyzed cleavage of cystine produce sulfane-sulfur donors for other sulfurtransferases, such as rhodanese [4–8]. Rhodanese subsequently utilizes cystathionase products as sulfane-sulfur substrates, transferring the sulfane-sulfur to CN, detoxifying CN via oxidation of SCN. The findings in this *in vivo* study, that inhibition of cystathionase increased the toxicity of CN, adds further support to the hypothesis that cystathionase-catalyzed reactions contributed to the sulfane-sulfur pool. Further studies, which measure the sulfane-sulfur pool size with varying degrees of

TABLE 1. Effect of propargylglycine on three liver enzymes

Propargylglycine (μmol/kg)	Cystathionase (μmol α-ketobutyrate/mg protein/min)	Rhodanese (μmol SCN/mg protein/min)	MST (μmol SCN/mg protein/min)
0	0.251 ± 0.030*	1.33 ± 0.23*	0.42 ± 0.04*
250	0.008 ± 0.003†	1.39 ± 0.23*	0.44 ± 0.04*

Values are means ± SD, N = 6.
*† Values with different symbols for the same enzyme are significantly different ($P \leq 0.05$).

cystathionase inhibition, are needed to provide more compelling evidence that cystathionase activity contributes to the sulfane-sulfur pool.

The purpose of this study was to determine if cystathionase participates in the detoxification of CN *in vivo*. The results indicate that the inhibition of cystathionase did increase the toxicity of CN, suggesting that cystathionase does participate in the endogenous CN detoxification process *in vivo*. The mechanism by which cystathionase contributes to CN detoxification *in vivo* was not determined in this study. With the determination that cystathionase does participate in the CN detoxification *in vivo*, future experiments designed to examine possible mechanisms of cystathionase action, and their quantitative contribution to CN detoxification, are needed in order to fully understand the role and significance of this enzyme in the endogenous CN detoxification system. Once the mechanism(s) by which cystathionase contributes to CN detoxification *in vivo* and its quantitative significance are better defined, it may be possible to develop new therapies for CN which utilize the action of cystathionase in the CN detoxification system.

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