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Inhibition of γ-glutamyl transpeptidase and induction of glutathionuria by γ-glutamyl amino acids

(glutathione/kidney/heavy metals/toxicity/leukotrienes)

MARY E. ANDERSON AND ALTON MEISTER

Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

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ABSTRACT **Treatment of mice with various γ-glutamyl amino acids leads to marked urinary excretion of glutathione and other γ-glutamyl compounds. There is good correlation between the affinity of γ-glutamyl transpeptidase for various γ-glutamyl amino acids and the extent of glutathionuria. The findings indicate that the administered γ-glutamyl compounds effectively compete with glutathione (exported from kidney cells and present in the glomerular filtrate) for the enzyme. The administration of certain γ-glutamyl amino acids appears to be a specific and nontoxic procedure for *in vivo* inhibition of γ-glutamyl transpeptidase that may be useful in experimental work on glutathione metabolism and function and also for treatment of certain toxicities and for modulation of the metabolism of endogenous glutathione conjugates.**

Studies on the metabolism of glutathione (GSH) indicate that GSH is exported from many types of cells (1, 2). GSH exported from the liver accounts for most of the blood plasma GSH, a large fraction of which is utilized by the kidney. However, this pathway of interorgan transport of GSH accounts for only some of the GSH that is exported from cells. Thus, export of GSH from the kidney is several times greater than that from liver into blood and bile. Such exported GSH is utilized within the kidney tubule by the actions of γ-glutamyl transpeptidase and dipeptidase.

Previous studies (3, 4) showed that administration of certain inhibitors of γ-glutamyl transpeptidase, such as L- (or D-) γ-glutamyl-(*o*-carboxy)phenylhydrazide, other hydrazides, 6-diazo-5-oxo-L-norleucine, and L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125), leads to substantial urinary excretion of GSH. The studies in which the isomers of γ-glutamyl-(*o*-carboxy)phenylhydrazide were injected into mice suggested that a large fraction of the urinary GSH arose from the kidney. Thus, when an inhibitor of GSH biosynthesis (prothionine sulfoximine) was given prior to injection of the hydrazide, much less GSH was found in the urine. However, in these studies, the level of GSH in liver decreased to some extent, so that it could not be excluded that some urinary GSH arises from the liver (3).

In the course of investigations on the transport of γ-glutamyl cysteine and related compounds into kidney (5), we observed that the urine of the treated mice contained significant amounts of GSH. We subsequently examined the effects of administering a variety of other γ-glutamyl compounds and found that many of these are also effective GSH analogs and thus induce glutathionuria that is often of the same order of magnitude as found after administration of the hydrazide transpeptidase inhibitors. The findings offer an apparently specific (and nontoxic) procedure for *in vivo* inhibition of γ-glutamyl transpeptidase that may be useful in experimental work and possibly also in therapy.

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EXPERIMENTAL PROCEDURES

Materials. Male Swiss Webster mice (20–27 g) were obtained from Taconic Farms (Germantown, NY) and Hilltop Labs (Philadelphia). γ-Glutamyl amino acids were prepared as described (6) or obtained from Vega–Fox (Tucson, AZ) and Sigma. Purity was checked by chromatography on a Durrum model 500 amino acid analyzer. Highly purified rat kidney γ-glutamyl transpeptidase (7) was a gift from S. S. Tate of this department.

Procedures. The mice were fed ad libitum. Their urinary bladders were emptied by gentle abdominal pressure prior to subcutaneous injection of the compounds, and urine was collected at 15-min intervals for 1 hr in a tube containing 5 μl of 50% (wt/vol) 5-sulfosalicylic acid. We occasionally found elevated plasma GSH levels after moderate abdominal pressure was applied to mice; for this reason, studies of urinary and plasma GSH levels were carried out on different groups of mice. Plasma, kidneys, and livers were obtained and processed as described (8, 9). Total GSH was determined by the glutathione disulfide (GSSG) reductase/5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) recycling assay (9–11).

The urine samples were treated with dithiothreitol (5 mM; pH 7.0; 1 hr), followed by derivatization with 2-vinylpyridine (12). The derivatized samples were chromatographed on the amino acid analyzer, using lithium citrate buffers. The elution times for the vinylpyridine derivatives of γ-glutamylglutathione, γ-glutamylcysteine, GSH, cysteinylglycine, and cysteine were 120, 163, 165, 220, and 235 min, respectively.

γ-Glutamyl transpeptidase was assayed using L-γ-glutamyl-*p*-nitroanilide; the rate of formation of *p*-nitroaniline at 37°C was measured at 405 nm (13). The reaction mixtures (final volume 1 ml) contained Tris·HCl (pH 7.5; 40 mM), L-γ-glutamyl-*p*-nitroanilide (30 and 60 μM), γ-glutamyl amino acid (30 and 150 μM), and the enzyme (0.115 unit). The reaction was initiated by adding enzyme. The absorbance scale was 0.02 or 0.05 to ensure that initial rates were obtained. The apparent hydrolysis K_i (K_{hi}) values were determined from a Dixon plot and normalized to the apparent K_m value for L-γ-glutamyl-*p*-nitroanilide (6 μM) (14).

RESULTS

Normal mouse urine contains <0.01 mM GSH. One hour after administration of γ-glutamylcysteine disulfide (4 mmol/kg of body weight), the urinary level of GSH was about 6 mM (Table 1). In a similar protocol (3), 3.6–5.6 mM GSH was found in the urine of mice injected with L-γ-glutamyl-(*o*-carboxy)phenylhydrazide. Administration of the hydrazide led to an ≈30% decrease in kidney GSH levels and to an ≈13% decrease in liver GSH levels. On the other hand, administration of γ-glutamylcysteine (or of related com-

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; AT-125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Table 1. Induction of glutathionuria by γ-glutamyl compounds

Treatment	Urinary GSH, mM	No. of mice
Saline (controls)	<0.01	>20
(L-γ-Glu-L-Cys)$_2$	5.77 ± 1.05	5
L-γ-Glu-L-(Cys)$_2$	2.63 ± 0.33	4
L-γ-Glu-L-Met	8.54 ± 0.99	4
L-γ-Glu-L-Glu	5.28 ± 1.05	4
L-γ-Glu-L-Ala	5.69 ± 1.26	3
L-γ-Glu-L-γ-Glu-L-Ala	5.07 ± 0.73	3
S-Methylglutathione	5.21 ± 1.30	4
L-γ-Glu-Gly-Gly	3.29 ± 1.16	8
L-γ-Glu-L-Tyr	3.19 ± 0.80	4
L-γ-Glu-Gly	2.67 ± 0.40	3
L-γ-Glu-L-Leu	0.60 ± 0.07	4
L-γ-Glu-L-α-Aba*	1.52 ± 0.38	3
L-γ-Glu-L-Gln	1.17 ± 0.55	9
L-γ-Glu-L-Ile	1.00 ± 0.10	4
L-γ-Glu-ε-L-Lys	0.77 ± 0.17	3
L-γ-Glu-L-Asp	0.26 ± 0.10	4
L-γ-Glu-L-Val	0.49 ± 0.25	3
D-γ-Glu-L-Met	0.18 ± 0.05	10
L-Cystine, L- (and D-) glutamine, L-glutamate plus L-cysteine, L-glutamate plus L-α-aminobutyrate, L-Glu-L-Tyr, L-Glu-L-Val, L-Glu-L-(α)-Lys, L-Glu-L-Ala	<0.03	3–4 (each)

Mice were injected subcutaneously with a 200 mM neutral solution of the indicated compound (dose, 4 mmol/kg of body weight) or with an equivalent volume of saline (0.9% NaCl). Urine was collected for 1 hr.
*Aba, α-aminobutyrate.

pounds) increases kidney GSH levels (5) and does not affect liver GSH levels. It was shown previously (5) that these compounds are effectively transported into kidney and used directly by glutathione synthetase for GSH synthesis, leading to elevated levels of GSH. In contrast, we found in the present studies that administration of other γ-glutamyl amino acids did not affect significantly the GSH levels of kidney or liver during the experimental period (1 hr). After administration of γ-glutamyl amino acid, the levels of GSH found in the blood plasma varied in different experiments from the normal range (20–40 μM) to about twice these values. The explanation for this variability is not apparent. However, the findings differ greatly from those of previous studies (3) in which administration of the hydrazide and other inhibitors of the transpeptidase led to marked increases in plasma GSH levels.

Previous studies (3, 15) showed that administration of either the hydrazide inhibitors of γ-glutamyl transpeptidase or of AT-125 induced urinary excretion of γ-glutamylcyst(e)ine. In the present study, we found γ-glutamylcysteine (as the vinylpyridine derivative) in the urine of mice that had been given γ-glutamyl compounds. After γ-glutamylglycylglycine was given, the urine contained γ-glutamylcysteine at levels that were about 10% those of glutathione. In these experiments and in studies in which γ-glutamylalanine was given, the urine also contained significant amounts of γ-glutamylglutathione (16) and of compounds that were eluted in the positions of several γ-glutamyl amino acids (but which were different from the administered γ-glutamyl compound). Analyses before and after reduction with dithiothreitol showed that a considerable amount of urinary GSH was in the form of mixed disulfides, especially that between GSH and cysteine, which is not a substrate of GSSG reductase. This indicates that the total excretion of GSH moieties is probably much greater, perhaps by as much as 100%, than indicated by the data obtained by the GSSG/Nbs$_2$ recycling assay (Table 1).

As indicated in Table 1, administration of the γ-glutamyl derivatives of methionine, glutamate, alanine, γ-glutamylalanine, glycylglycine, tyrosine, glycine, and leucine was followed by substantial urinary excretion of GSH. The amounts of GSH excreted in these studies are estimated to be in the range 3–6 μmol, which is a significant fraction, perhaps 50–80%, of the total kidney tubular burden of GSH. We estimate that the amount of GSH excreted during the experimental period can be as high as 5–8% of the total body GSH. Other γ-glutamyl amino acids (Table 1) also induced glutathionuria, whereas several α-glutamyl amino acids, free amino acids, and mixtures of glutamate and cysteine (or α-aminobutyrate) did not.

Although it is known that various γ-glutamyl compounds can interact with γ-glutamyl transpeptidase, detailed studies on the affinity of this enzyme for γ-glutamyl amino acids have apparently not been reported. We therefore estimated the apparent K_{hi} values for a number of γ-glutamyl amino acids, using a purified transpeptidase preparation. These values are plotted in Fig. 1 against those for urinary GSH obtained in the *in vivo* studies, and a fairly close correlation is apparent. The apparent affinity of the enzyme for γ-glutamyl amino acid substrates varies over an ≈6-fold range. In contrast, the affinity for the corresponding free amino acids varies over a range of several hundred (17), suggesting that the γ-glutamyl moiety contributes significantly to binding. For example, such amino acids as aspartate, lysine, and valine are extremely poor acceptor substrates, whereas the corresponding γ-glutamyl amino acids exhibit significant interaction with the enzyme. The data also indicate that glutamine, a very poor γ-glutamyl substrate of the enzyme, is ineffective in inducing glutathionuria. γ-Glutamyl-γ-glutamylalanine, which induced glutathionuria (Fig. 1), is probably rapidly converted *in vivo* to γ-glutamylalanine, which has a much higher affinity for the enzyme.

DISCUSSION

The findings may be explained in terms of competition for γ-glutamyl transpeptidase between the administered GSH

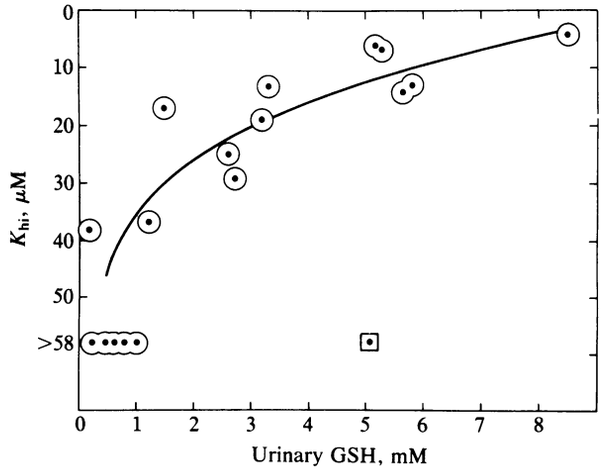

FIG. 1. Correlation between urinary GSH excretion (GSH + 2 GSSG) and the apparent K_{hi} values of γ-glutamyl compounds for γ-glutamyl transpeptidase. Urinary GSH and the apparent K_{hi} values were determined as described in *Experimental Procedures*. Points (left to right), >58 μM: γ-Glu-Val, γ-Glu-Asp, γ-Glu-ε-Lys, γ-Glu-Ile, γ-Glu-Leu, γ-Glu-γ-Glu-Ala (in square). Other points (left to right): D-γ-Glu-Met, γ-Glu-Glu, γ-Glu-α-aminobutyrate (γ-Glu-Aba), γ-Glu-(Cys)$_2$, γ-Glu-Gly, γ-Glu-Tyr, γ-Glu-Gly-Gly, *S*-methyl-GSH, γ-Glu-Glu, γ-Glu-Ala, (γ-Glu-Cys)$_2$, γ-Glu-Met.

analog and GSH, most of which is normally exported from renal cells into the tubule. The amounts of GSH found in the urine in these studies reflect the very substantial output of GSH by renal cells and strongly support the view that the kidney functions in a quantitatively significant cycle of GSH transport and metabolism. Renal tubular GSH is effectively utilized by γ-glutamyl transpeptidase and dipeptidase, and GSH is also effectively metabolized basolaterally (18). Thus, the substantial output of GSH by the kidney plus GSH derived from the blood plasma is matched by efficient utilization of GSH in the tubule and in the basolateral circulation, so that very little GSH reaches the urine and the renal venous plasma.

Administration of γ-glutamyl amino acids seems to be a useful procedure for inhibiting γ-glutamyl transpeptidase *in vivo*. Although γ-glutamylcysteine and closely related compounds increase kidney levels of GSH (5), the γ-glutamyl derivatives of the other amino acids studied did not affect kidney GSH levels significantly during the experimental period. We found no effect of γ-glutamyl amino acids on liver GSH levels, nor was any toxicity apparent. Administration of γ-glutamyl amino acids probably also inhibits γ-glutamyl transpeptidase in other tissues but does not produce other serious perturbation of metabolism. This is consistent with the finding of elevated plasma GSH levels in some experiments. Although the isomers of γ-glutamyl-(*o*-carboxy)-phenylhydrazide are good inhibitors of the transpeptidase, they are split to a slight extent by this enzyme, leading to formation of *o*-carboxyphenylhydrazine, which is toxic; doses of more than about 2 mmol per kilogram of body weight are often fatal to mice. Another complication is formation of GSH conjugates of the inhibitor and its metabolic products (19). Although AT-125 is a potent transpeptidase inhibitor, this compound also inhibits a number of other enzymes, notably the glutamine amidotransferases (20, 21).

The present approach offers an apparently selective procedure for *in vivo* inhibition of γ-glutamyl transpeptidase. Its effectiveness is evident from the extent of glutathionuria achieved with rather modest doses of γ-glutamyl amino acids. In addition to competitive inhibition of the transpeptidase, it is to be expected that high levels of an administered γ-glutamyl amino acid would inhibit transport of other γ-glutamyl amino acids into the kidney. Indeed, as noted previously (3, 4, 22), high extracellular levels of GSH also have this effect which probably accounts for the appearance of large amounts of γ-glutamyl cysteine and other γ-glutamyl amino acids in the urine. Further studies on this phenomenon are required. In addition, studies on the effects of longer-term administration of γ-glutamyl amino acids are needed.

In addition to the potential usefulness of this approach in *in vivo* studies on γ-glutamyl transpeptidase and GSH metabolism, the availability of a method for inhibiting the transpeptidase that would be expected to be nontoxic suggests therapeutic applications. Thus, increased extracellular GSH levels in the kidney might be of value in the treatment of toxicity produced by heavy metals and nephrotoxic drugs. This might also be achieved by giving glutathione itself, whose administration is followed by significant glutathionuria (23), or by giving glutathione monoesters (24). The present studies indicate that non-sulfur-containing γ-glutamylcompounds may also be useful. Since γ-glutamyl amino acids are effectively transported into the kidney, the possibility of using mixtures of γ-glutamyl amino acids might be considered so as to avoid the occurrence of amino acid imbalances.

The urinary excretion of methylmercury by intoxicated rats was reported to be increased 33-fold after AT-125 was given (25); this effect is presumably related to complex formation between methylmercury and GSH. In an approach to elimination of selenium, bromobenzene was administered to dogs fed toxic amounts of selenium (26), to steers that had grazed on a seleniferous range (26), and to a human (27) to facilitate removal of selenium as the corresponding selenomercapturic acid. Later studies on rabbits failed to show "pronounced amounts" of selenium in this mercapturate, and the use of bromobenzene was decried because of its toxicity (28). Induction of glutathionuria might serve to remove selenium present in selenocysteine moieties. Inhibition of γ-glutamyl transpeptidase would be expected to significantly influence metabolism of GSH conjugates both of exogenous and of endogenous origin. In certain types of toxicity, it may be desirable to decrease such metabolism. Thus, AT-125 (as well as buthionine sulfoximine) decreased the toxicity of methyl chloride to mice (29). Finally, we suggest that the present approach may be useful, at least as an experimental tool, if not also in therapy, for modification of the metabolism of compounds in the leukotriene series (30). It would be anticipated that inhibition of γ-glutamyl transpeptidase, which would block formation of leukotrienes of type D from type C, would have significant physiological effects under appropriate conditions.

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