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Identification of the Mixed Disulfide of Glutathione and Cysteinylglycine in Bile: Dependence on γ-Glutamyl Transferase and Responsiveness to Oxidative Stress[1]

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ABSTRACT

Biliary excretion of glutathione disulfide (GSSG) is used as an index of oxidative stress. Analysis of endogenous thiols and disulfides in rat bile by reverse phase high performance liquid chromatography with electrochemical detection revealed an unknown disulfide which eluted immediately after GSSG. This disulfide was tentatively identified as the mixed disulfide of glutathione (GSH) and cysteinylglycine (Cys-Gly), based on its coelution on a reverse phase column with the synthetic GS-Cys-Gly. GS-Cys-Gly was also detected in bile of other species. On analyzing species differences in biliary excretion of GSH-related thiols and disulfides, it was concluded that biliary excretion of GS-Cys-Gly was related to the excretion of both GSSG and Cys-Gly, which is formed from GSH by γ-glutamyltransferase (γ-GT)-catalyzed hydrolysis. Species with low hepatic γ-GT (*i.e.*, hamsters and mice) excreted little Cys-Gly in bile. These animals excreted negligible amounts of GS-Cys-Gly even when biliary excretion of GSSG was markedly increased by paraquat-induced oxidative stress. Rats and guinea pigs, which have high hepatic γ-GT activities, excreted large amounts of both Cys-Gly and GS-Cys-Gly. Treatment of rats with acivicin, an inhibitor of γ-GT, decreased the biliary excretion of both Cys-Gly and GS-Cys-Gly. Paraquat treatment of rats resulted in an increase in GSSG excretion with concomitant increase of GS-Cys-Gly excretion. Rabbits, which also have high hepatic γ-GT activity, excreted little GS-Cys-Gly into bile. This may be due to the fact that the hepatobiliary transport of GSH and GSSG is very low in rabbits. However, when the biliary excretion of GSSG was increased by paraquat administration, rabbits excreted large amounts of GS-Cys-Gly, even exceeding that of GSSG. These observations suggest that biliary GS-Cys-Gly originates from a thiol-disulfide exchange reaction between GSSG and Cys-Gly, with the latter being formed by γ-GT-catalyzed hydrolysis of GSH. Our results with paraquat confirm that increased biliary excretion of GSSG is a good indicator of oxidative stress in mice and hamsters (*i.e.*, in species with low activity of hepatic γ-GT). However, in species with high hepatic γ-GT (*i.e.*, rats and rabbits), the biliary excretion of GS-Cys-Gly may be supplemental or even superior to the biliary excretion of GSSG to indicate oxidative stress *in vivo*.

GSH (γ-glutamylcysteinylglycine) is the major intracellular nonprotein thiol (Meister and Anderson, 1983). GSH plays an important role in maintenance of intracellular redox state, detoxification of electrophiles and reduction of peroxides and free radicals (Kaplowitz, 1981; Kosower and Kosower, 1978). GSH is primarily synthesized in the liver, from where it is transported into both the circulation and the bile (Eberle *et al.*, 1981; Sies *et al.*, 1979). The bile canalicular enzyme, γ-GT, initiates the hydrolysis of GSH to other sulfhydryls (*i.e.*, Cys-Gly and Cys), which are also excreted in bile. In addition to sulfhydryls (*i.e.*, GSH, Cys-Gly and Cys), their disulfides are also found in bile (Abbott and Meister, 1986; Stein *et al.*, 1986). These include GSSG, Cys, Cys-Gly-DS and mixed disulfide of GSH and Cys (GS-Cys). During oxidative stress, hepatic concentration of GSSG is elevated and the GSSG is preferentially transported into bile. Therefore, biliary excretion of GSSG has been used as an index of oxidative stress (Akerboom *et al.*, 1982; Lauterburg *et al.*, 1984). In this article, we describe a hitherto unknown biliary disulfide that was found in bile of various animal species. The present study aimed to identify this compound, clarify its relationship with GSH and determine what factors affect its formation and biliary excretion. The factors considered included hepatic activity of γ-GT and oxidative stress. The latter condition was induced by treatment with paraquat, a herbicide which exerts its toxic effect *via* generation of reactive oxygen species (Bus and Gibson, 1976).

Materials and Methods

Chemicals. Cys, Dowex-1-chloride, reduced and oxidized GSH, paraquat and H_2O_2 were obtained from Sigma Chemical Co. (St. Louis,

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ABBREVIATIONS: Cys, cysteine; Cys-Gly, cysteinylglycine; Cys-Gly-DS, cysteinylglycine disulfide; GS-Cys-Gly, mixed disulfide of glutathione and cysteinylglycine; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; γ-GT, γ-glutamyl transferase.

MO). L-Cysteinyl-bis-glycine was obtained from Chemical Dynamics Corporation (South Plainfields, NJ). 1-Heptanesulfonic acid and monochloroacetic acid were from Kodak (Rochester, NY), and ultrapure *N,N*-dimethylformamide and ultrapure perchloric acid (70%) were from Alfa Products (Danvers, MA). HPLC-grade methanol was obtained from Fisher Scientific (St. Louis, MO).

Synthesis of GS-Cys-Gly. GS-Cys-Gly was synthesized based on the principle described by Eriksson and Eriksson (1967). The thiol sulfonate analogue of L-cysteinyl-bis-glycine was prepared according to Emiliozzi and Pichat (1959). To an excess of thiol sulfonate (about 0.5 mmol), 0.147 mmol of GSH were added in 4.5 ml of 0.01 M formic acid, the mixture was thoroughly stirred for a period of 120 min and the solid material was removed by centrifugation. The supernatant from the reaction mixture and the combined washings were applied to a Dowex 1-formate (X2, 50–100 mesh) column (4 × 15 cm). Subsequently, the column was washed with 1400 ml of H_2O followed by 0.15 M formic acid. After starting the elution with formic acid, 10-ml fractions were collected. Each fraction was analyzed by HPLC with electrochemical detection in order to identify the fractions which contained the synthesized compound. The fractions which contained a single peak were combined and concentrated on a rotary evaporator at 23°C. Thereafter, the solution was lyophilized. Amino acid analysis after acid hydrolysis of the synthesized compound indicated that it was composed of Gly, Cys and glutamic acid in a molar ratio of 2:2:1.

Animals. Male CF_1 mice (30–35 g), golden Syrian hamsters (90–110 g), Sprague-Dawley rats (200–225 g), Hartley albino guinea pigs (300–350 g) and New Zealand White rabbits (1500–1800 g) were obtained from Sasco Inc. (Omaha, NE). They were provided tap water and lab chow (Ralston Purina Co., St. Louis, MO) *ad libitum* and were housed at 23–27°C on a 12-hr light/dark cycle.

Animal experiments. Rats, mice and hamsters were anesthetized with urethane (1.2 g/kg, i.p.). Rabbits were anesthetized with pentobarbital (30 mg/kg, i.p., plus 15 mg/kg, i.v.). Guinea pigs, which do not tolerate pentobarbital well, were anesthetized with a mixture of ketamine (Ketalar; Parke-Davis, Morris Plains, NJ; 100 mg/kg, i.p.) and xylazine (Rompun, Mobay Corporation, Animal Health Division, Shawnee, KS; 10 mg/kg, i.p.).

After median laparotomy, the common bile ducts in mice, hamsters, rats and guinea pigs were cannulated using 30-, 27-, 25- and 21-gauge needles, respectively, attached to an appropriate diameter polyethylene tubing. The common bile duct in rabbits was cannulated through an incision with polyethylene tubing (PE-100). The cystic duct in guinea pigs and rabbits was ligated before bile duct cannulation.

Bile was collected into 0.5 ml of 0.25 M perchloric acid for 20 min before and for six 20-min intervals after administration of paraquat. Paraquat was dissolved in saline and injected i.v. to rats, hamsters and rabbits at a dosage of 90 mg/kg and to mice at a dosage of 20 mg/kg.

Analytical methods. GSH, Cys, Cys-Gly, GSSG, Cys-Gly-DS and GS-Cys-Gly (which is identified in this study) were separated by HPLC on a reverse phase column at 35°C using a mobile phase of 0.1 M monochloroacetic acid, 2.5 mM 1-heptanesulfonic acid (pH 2.60), methanol and *N,N*-dimethylformamide (96.5:3:0.5 v/v) at a flow rate of 1.0 ml/min (Stein *et al.*, 1986). The mobile phase was purged of oxygen by sparging with helium gas and continuous refluxing at 50–55°C. Reduced sulfhydryls were detected by their electrochemical oxidation using a mercury-gold electrode (W2; 0.15 V). Oxidized sulfhydryls were first reduced by another mercury-gold electrode (W1; −1.0 V) located upstream from the W2 electrode.

Bile samples collected into perchloric acid were diluted appropriately with 0.25 M perchloric acid before analysis. Standard solutions containing known amounts of thiols and disulfides in 0.25 M perchloric acid were analyzed each day along with bile samples. All samples were kept at −80°C before analysis.

Results

Identification of GS-Cys-Gly in rat bile. GSH and its metabolites in rat bile were analyzed by HPLC with electrochemical detection. A representative chromatogram of a bile sample is shown in figure 1A. The elution order of GSH and its metabolites was as follows: Cys, GSH, Cys-Gly, GSSG, followed by an unknown peak and Cys-Gly-DS. Because the unknown compound eluted immediately after GSSG, it was suspected to be a disulfide. In the process of electrochemical detection, thiols are detected by their electrochemical oxidation. In order to detect the disulfides, they are first reduced to thiols by a mercury-gold electrode (W1; −1.0 V) located upstream from the W2 electrode (Stein *et al.*, 1986). When reduction of disulfides was prevented by turning off the electrical potential on the upstream electrode, the thiols (GSH, Cys, Cys-Gly) are detected, but the disulfides (GSSG and Cys-Gly-DS) are not (fig. 1B). Interestingly, the unknown compound also became undetectable. This observation confirmed that the unknown peak represents a disulfide. Because it was thought that the unknown disulfide is GS-Cys-Gly, this compound was synthesized and analyzed by HPLC. The retention times of the synthesized GS-Cys-Gly (fig. 1C) and the unknown disulfide in the bile sample (fig. 1A) were identical. Therefore, the unknown disulfide is tentatively identified as GS-Cys-Gly.

Dependence of the biliary excretion of GS-Cys-Gly on hepatic γ-GT activity. The role of hepatic γ-GT in the biliary excretion of GS-Cys-Gly was assessed in two ways: by examining the effect of acivicin, a potent inhibitor of γ-GT, and by comparing various animal species with different hepatic γ-GT activities. The effect of acivicin on the biliary excretion of GSH and its hydrolysis products in rats is shown in table 1. Acivicin administration (100 μmol/kg, i.v.), which reduces the hepatic γ-GT activity by 88% (Gregus *et al.*, 1987a, b), decreased the biliary excretion of GSH hydrolysis products (*i.e.*, Cys, Cys-Gly and Cys-Gly-DS by 40, 57 and 80%, respectively), while increasing the excretion of GSH and GSSG by 5 and 40%, respectively; these increases were not statistically significant. Inhibition of γ-GT also diminished the biliary excretion of GS-Cys-Gly by 34% (table 1).

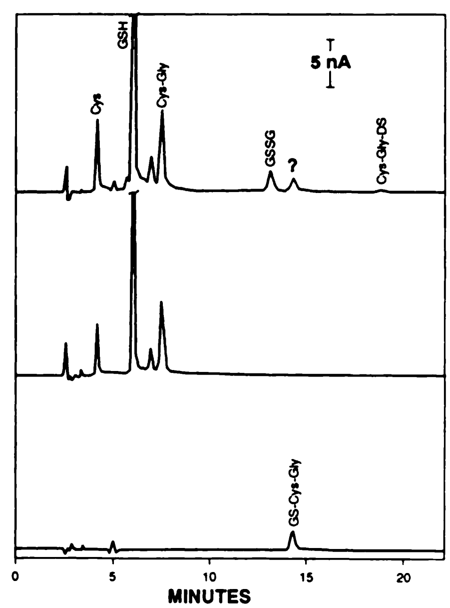

Fig. 1. Identification of GS-Cys-Gly in rat bile by HPLC with electrochemical detection. Top panel: A typical chromatogram of thiols and disulfides in rat bile. Middle panel: Chromatogram of thiols in bile after turning off the reducing potential (-I V). Bottom panel: Chromatogram of GS-Cys-Gly standard (500 pmol).

TABLE 1

Effect of acivicin on biliary excretion of GSH and its hydrolysis products

Bile was collected for 20 min before and at 100 to 120 min after acivicin (100 μmol/kg, i.v.) administration. GSH and its hydrolysis products were measured in bile. Values are means ± S.E. of three rats.

Metabolite	Biliary Excretion		Percent Control
	Control	Acivicin	
	nmol/min/kg b.wt.		
Cys	10.61 ± 1.86	6.04 ± 2.18[a,b]	57
Cys-Gly	37.73 ± 5.88	16.80 ± 2.57[b]	44
Cys-Gly-DS	4.86 ± 0.66	1.08 ± 0.33[b]	22
GS-Cys-Gly	15.16 ± 2.10	10.46 ± 1.92[b]	68
GSH	86.76 ± 23.29	91.73 ± 8.31	106
GSSG	18.93 ± 4.99	25.46 ± 4.43	134

[a] Data was analyzed by Student's *t* test.
[b] Significantly different ($P < .05$) from controls.

Biliary excretion rates of GSH and its metabolites, including the newly identified GS-Cys-Gly, in hamsters, mice, rats, rabbits and guinea pigs. These species are different with respect to both the biliary excretion of total nonprotein sulfur ($\sum S$) and the composition of thiols and disulfides in bile (table 2). Mice and rats excreted more than 200 nmol sulfur/min/kg, followed by guinea pigs and hamsters, whereas rabbits excreted only 20 nmol/min/kg. In mice and hamsters, whose hepatic γ-GT activity is low (table 2), biliary thiols and disulfides were composed mainly of GSH and GSSG. These two species excreted only negligible amounts of GS-Cys-Gly, representing only approximately 1% of total biliary nonprotein sulfur. In contrast, in rats, rabbits and guinea pigs, species with higher hepatic γ-GT activity (table 2), the biliary thiols and disulfides were composed mainly of GSH hydrolysis products in reduced and oxidized forms (table 2). Biliary excretion rate of GS-Cys-Gly was highest in rats, lower in guinea pigs and very low in rabbits. Nevertheless, in these three species, excretion of GS-Cys-Gly-derived sulfur represented more than 10% of the total biliary sulfur excretion.

Effect of oxidative stress on the biliary excretion of GS-Cys-Gly. The time course of the biliary excretion of GSSG and GS-Cys-Gly during paraquat-induced oxidative stress in mice, hamsters, rats and rabbits is shown in figure 2. Marked species variations in the biliary excretion of GSSG and GS-Cys-Gly excretion to paraquat were observed. Injection of paraquat to hamsters or mice, species with low γ-GT activity, markedly increased the biliary excretion of GSSG. Within 20 to 40 min after paraquat administration, the biliary excretion of GSSG increased to 95 nmol/min/kg in hamsters and 440 nmol/min/kg in mice. Thereafter, the rate of GSSG excretion was fairly constant in hamsters, but gradually declined in mice (fig. 2, panel 1 and 2 from top). In contrast, the maximal biliary excretion rate of GS-Cys-Gly in hamsters and mice did not exceed 1 and 10 nmol/min/kg, respectively, after paraquat administration. Cys-Gly-DS was not detected in either mouse or hamster bile.

Biliary excretion of GSSG, GS-Cys-Gly and Cys-Gly-DS after paraquat administration to rats, a species with high γ-GT activity. Paraquat administration to rats increased the biliary excretion of GSSG and GS-Cys-Gly 3- and 2-fold, respectively, but did not influence the excretion of Cys-Gly-DS (fig. 2, panel 3 from top). The maximal rates of both GSSG and GS-Cys-Gly excretion were observed 60 to 80 min after paraquat administration. Thereafter, the rates of biliary excretion for both studies were fairly constant throughout the 2-hr bile collection.

The effect of paraquat on the biliary excretion of GS-Cys-Gly and GSSG in rabbits, a species with high γ-GT activity and low sulfur excretion into bile. Rabbits responded to paraquat (bottom panel) with increased biliary excretion of Cys-Gly-DS, GS-Cys-Gly and GSSG. In absolute terms, the excretion rate of Cys-Gly-DS exceeded those of GS-Cys-Gly and GSSG; however, the relative increases in biliary excretion of Cys-Gly-DS and GS-Cys-Gly after paraquat administration were comparable and exceeded that of GSSG.

Discussion

A number of xenobiotics are thought to exert their toxic effects by generation of reactive oxygen species (Bus and Gibson, 1976). Hydrogen peroxide, one of the reactive oxygen species, may be detoxified by GSH peroxidase, resulting in formation of GSSG from GSH. As a result, the biliary excretion of GSSG substantially increases after administration of various chemicals which induce oxidative stress, including paraquat (Bregelius and Anwer, 1981; Lauterburg *et al.*, 1984), diquat (Smith, 1987), and *t*-butyl hydroperoxide (Akerboom *et al.*, 1982; Lauterburg *et al.*, 1984). Therefore, biliary excretion of GSSG has been used as an index of oxidative stress (Lauterburg *et al.*, 1984).

In the present study, a new disulfide was found in bile and identified as GS-Cys-Gly. Wikberg (1953) reported production of GS-Cys-Gly during hydrolysis of GSH *in vitro*. However, formation of GS-Cys-Gly *in vivo* has not been previously noted. GS-Cys-Gly may be formed in various ways. In theory, GS-Cys-Gly could be formed in the liver as a result of a reaction between Cys-Gly and GSH or GSSG, or as a product of γ-GT-catalyzed hydrolysis of GSSG. However, intrahepatic formation

TABLE 2

Species differences in hepatic γ-GT activity, bile flow and biliary excretion rates of GSH-related thiols and disulfides

Values are means ± S.E. of five to seven animals. N.D., not detected

Species	Hepatic γ-GT Activity[a]	Bile Flow	Biliary Excretion						
			$\sum S$[b]	GSH	Cys-Gly	Cys	GSSG	Cys-Gly-DS	GS-Cys-Gly
	nmol/min/g	*μl/min/kg*	*nmol/min/kg*						
Hamsters	17.1 ± 5.2	58 ± 17	140 ± 27	75.4 ± 13	1.0 ± 0.3	0.25 ± 0.08	31.0 ± 6.9	N.D.	1.14
Mouse	23.4 ± 1.4	90 ± 6.2	390 ± 38	271 ± 27	4.69 ± 0.9	2.79 ± 0.4	54.6 ± 7.4	N.D.	1.86 ± 0.3
Rat	201 ± 37	82 ± 4.9	243 ± 33	97.2 ± 15	51.5 ± 4.7	14.5 ± 3.3	17.7 ± 4.4	280 ± 1.0	19.6 ± 4.2
Rabbit	350 ± 20	67 ± 31	18 ± 2.9	0.20 ± 0.1	0.25 ± 0.1	0.03 ± 0.01	0.26 ± 0.2	7.6 ± 1.4	1.53 ± 1.3
Guinea pig	939 ± 63	225 ± 37	137 ± 21	24.0 ± 10	68.2 ± 7.9	0.56 ± 0.9	1.28 ± 0.88	11.6 ± 6.8	9.3 ± 5.0

[a] Data from Gregus *et al.* (1988).
[b] Represents the sum of GSH + Cys-Gly + Cys + 2 GSSG + 2 Cys-Gly-DS + 2 GS-Cys-Gly.

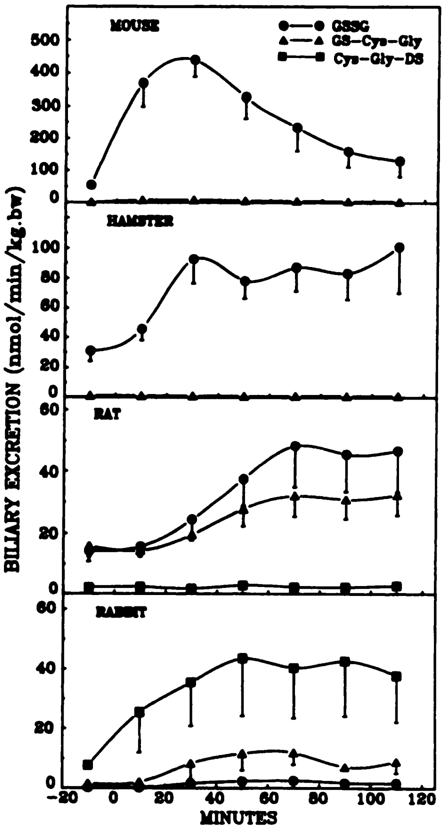

Fig. 2. Effect of paraquat on the biliary excretion of GSSG, GS-Cys-Gly (not detected in mice and hamsters) and Cys-Gly-DS in mice, hamsters, rats and rabbits. Paraquat was administered (i.v.) at 0 time. Dosage regimens were given in materials and methods. Each symbol represents the mean ± S.E. of four to seven animals.

of GS-Cys-Gly appears unlikely, because the concentration of Cys-Gly is very low in the liver (Gregus *et al.*, 1987a, b) and the catalytic site of γ-GT is not intracellular (Horiuchi *et al.*, 1978). Therefore, intrabiliary formation of GS-Cys-Gly is more likely.

γ-GT is located in the canalicular membrane with its catalytic site facing the lumen (Albert *et al.*, 1964; Inoue *et al.*, 1983; Szewczuk *et al.*, 1980). Thus, γ-GT could hydrolyze GSSG transported from the liver to the bile to form GS-Cys-Gly. However, GSSG is a relatively poor substrate for γ-GT (Tate and Meister, 1974). Therefore, the following mechanism of GS-Cys-Gly formation appears more likely. GSH transported into bile undergoes partial hydrolysis, catalyzed by γ-GT to form Cys-Gly. The resultant Cys-Gly may enter into a thiol-disulfide exchange reaction with GSSG. In this reaction, Cys-Gly would reduce GSSG by forming GS-Cys-Gly and GSH. Reduction by a thiol is known to proceed through dissociation of the thiol group to form a thiolate anion (Benesch and Benesch, 1955; Jocelyn, 1987). The dissociation constant (*pK*) of the thiol group in Cys-Gly is 7.87 (Benesch and Benesch, 1955), the lowest of all GSH-related thiols. This implies that at the pH of bile (approximately 7.8), half of the Cys-Gly molecules form thiolate anions and, thus, can readily reduce disulfides, such as GSSG, with the resultant formation of a mixed disulfide. Thus, formation of GS-Cys-Gly by thiol-disulfide exchange between Cys-Gly and GSSG is feasible. Alternatively, GS-Cys-Gly may be formed in bile by disulfide formation, which also proceeds through ionization of the thiol group, between GSH and Cys-Gly.

Intrabiliary formation of GS-Cys-Gly by thiol-disulfide exchange reaction would imply that the formation of GS-Cys-Gly would depend on the biliary concentration of both Cys-Gly and GSSG. The concentration of Cys-Gly in bile would depend on the rate of hepatobiliary transport of GSH and the efficacy of GSH hydrolysis by γ-GT, whereas the biliary concentration of GSSG would be dependent on the rate of hepatobiliary transport of GSH or GSSG, the latter being related to the extent of intrahepatic oxidation of GSH to GSSG. The following observations confirm these implications and support the above described mechanism for formation of GS-Cys-Gly. 1) Inhibition of hepatic γ-GT decreased the biliary Cys-Gly and diminished biliary excretion of GS-Cys-Gly in rats (table 1). 2) Species with low hepatic γ-GT (*i.e.*, hamsters and mice) excreted little Cys-Gly in bile (table 2). These species excreted negligible amounts of GS-Cys-Gly (table 2), even when biliary excretion of GSSG was markedly increased by paraquat-induced oxidative stress (fig. 2). 3) Rats and guinea pigs, which have relatively high hepatic γ-GT activity, excreted large amounts of both Cys-Gly and GS-Cys-Gly (table 2). 4) Paraquat treatment of rats resulted in increases in GSSG excretion with concomitant increase of GS-Cys-Gly excretion (fig. 2). 5) Paradoxically, rabbits excreted little GS-Cys-Gly in bile despite their high hepatic γ-GT activity (table 2). This may be due to the fact that the hepatobiliary transport of GSH and GSSG, as reflected by the biliary excretion of total nonprotein thiols ($\sum$S), is very low (table 2). The high γ-GT activity combined with the low hepatobiliary transport of GSH and GSSG (*i.e.*, low rate of substrate supply for γ-GT) and relatively slow bile flow (table 2; *i.e.*, long reaction time for the γ-GT catalyzed hydrolysis of GSH), would result in rapid and almost complete hydrolysis of GSH to Cys-Gly, which leaves little GSH or GSSG to react with Cys-Gly to form the mixed disulfide. Instead, Cys-Gly will react with itself, forming large amounts of Cys-Gly-DS (table 2). However, when biliary excretion of GSSG was increased by paraquat treatment, rabbits also excreted considerable amounts of GS-Cys-Gly (fig. 2). This finding supports our hypothesis that the low amount of GS-Cys-Gly in rabbit bile in resting state is due to unavailability of GSSG for the thiol-disulfide exchange reaction. Interestingly, paraquat also induced a large increase in biliary excretion of Cys-Gly-DS in rabbits (fig. 2), which may also be secondary to enhanced excretion of GSSG. Cys-Gly-DS may be formed by sequential hydrolysis of the glutamate moieties of GSSG by γ-GT, with the intermediary formation of GS-Cys-Gly. Alternatively, as a result of the thiol disulfide exchange between GSSG and Cys-Gly, GSH can be released, which could give rise to Cys-Gly-DS *via* γ-GT-catalyzed hydrolysis and subsequent oxidation.

Identification of the new disulfide, GS-Cys-Gly, in bile further underlines the complexity of the metabolism of biliary GSH and the interaction of GSH hydrolysis products. In addition, discovery of GS-Cys-Gly in bile may have practical importance in toxicology as well. Increased excretion of GSSG in bile has been considered a major index for oxidative stress *in vivo*. Our results with paraquat confirm that in mice and hamsters (*i.e.*, in species with low activity of hepatic γ-GT), enhanced biliary excretion of GSSG is a good indicator of oxidative stress (fig. 2). However, rats responded to paraquat administration with similar increases in biliary excretion of both GSSG and GS-Cys-Gly (fig. 2), whereas in rabbits, increases in excretion of GS-Cys-Gly exceeded those of GSSG (fig. 2). Therefore, these observations indicate that biliary excretion of GS-Cys-Gly in species with high hepatic γ-GT activity may be supplementary or even superior to the biliary excretion of GSSG to predict oxidative stress *in vivo*.

References

ABBOTT, W. A. AND MEISTER, A.: Intrahepatic transport and utilization of biliary glutathione and its metabolites. Proc. Natl. Acad. Sci. USA **83:** 1246–1250, 1986.

AKERBOOM, T. P. M., BILZER, M. AND SIES, H.: Competion between transport of glutathione disulfide (GSSG) and glutathione S-conjugates from perfused rat liver into bile. FEBS Lett. **140:** 73–76, 1982.

ALBERT, Z., ORLOWSKA, J., ORLOWSKI, M. AND SZEWCZUK, A.: Histochemical and biochemical investigations of gamma-glutamyl transpeptidase in the tissues of man and laboratory rodents. Acta Histochem. **28:** 78–89, 1964.

BENESCH, R. E. AND BENESCH, R.: The acid strength of the -SH group in cysteine and related compounds. J. Am. Chem. Soc. **77:** 5877–5881, 1955.

BREGELIUS, R. AND ANWER, M. S.: Increased biliary GSSG-secretion and loss of hepatic glutathione in isolated perfused rat liver after paraquat treatment. Res. Commun. Chem. Pathol. Pharmacol. **31:** 493–502, 1981.

BUS, J. S. AND GIBSON, J. E.: Paraquat toxicity: Proposed mechanisms of toxicity involving lipid peroxidation. Environ. Health Perspect. **16:** 139–146, 1976.

EBERLE, D., CLARKE, R. AND KAPLOWITZ, N.: Rapid oxidation *in vitro* of endogenous and exogenous glutathione in bile of rats. J. Biol. Chem. **256:** 2115–2117, 1981.

EMILIOZZI, R. AND PICHAT, L.: Méthode simple de préparation d'acide L (+) amino-2 sulfino-3 propionique (acide cystéine sulfinique). Bull. Soc. Chim. France, 1887–1888, 1959.

ERIKSSON, B. AND ERIKSSON, S. A.: Synthesis and characterization of the L-cysteine-glutathione mixed disulfide. Acta Chem. Scand. **21:** 1304–1312, 1967.

GREGUS, Z., STEIN, A. F. AND KLAASSEN, C. D.: Effect of inhibition of γ-glutamyl transpeptidase on biliary and urinary excretion of glutathione-derived thiols and methylmercury. J. Pharmacol. Exp. Ther. **242:** 27–32, 1987a.

GREGUS, Z., STEIN, A. F. AND KLAASSEN, C. D.: Age-dependent biliary excretion of glutathione-related thiols in rats: Role of γ-glutamyl transferase. Am. J. Physiol. **253:** G 86–92, 1987b.

HORIUCHI, S., INOUE, M. AND MORINO, Y.: Gamma-glutamyl transpeptidase: Sideness of its active site on renal brush border membrane. Eur. J. Biochem. **87:** 429–437, 1978.

INOUE, M., KINNE, R., TRAN, T., BIEMPICA, L. AND ARIAS, I. M.: Rat liver canalicular membrane vesicles. Isolation and topological characterization. J. Biol. Chem. **258:** 5183–5188, 1983.

JOCELYN, P. C.: Chemical reduction of disulfides. *In* Methods in Enzymology, ed. by B. N. Jakoby and O. Griffith, vol. 143, pp. 246–265, Academic Press, New York, 1987.

KAPLOWITZ, N.: The importance and regulation of hepatic glutathione. Yale J. Biol. Med. **54:** 497–502, 1981.

KOSOWER, N. S. AND KOSOWER, E. M.: The glutathione status of cells. Int. Rev. Cytol. **54:** 109–160, 1978.

LAUTERBURG, B. H., SMITH, C. V., HUGHES, H. AND MITCHELL, J. R.: Biliary excretion of glutathione and glutathione disulfide in the rat. Regulation and response to oxidative stress. J. Clin. Invest. **73:** 124–133, 1984.

MEISTER, A. AND ANDERSON, M. E.: Glutathione. Ann. Rev. Biochem. **52:** 711–760, 1983.

SIES, H., KOCH, D. R., MARTINO, E. AND BOVERIES, A.: Increased biliary glutathione disulfide release in chronically ethanol-treated rats. FEBS Lett. **103:** 287–290, 1979.

SMITH, C. V.: Effect of BCNU pretreatment on diquat-induced oxidative stress and hepatotoxicity. Biochem. Biophys. Res. Commun. **144:** 415–421, 1987.

STEIN, A. F., DILLS, R. L. AND KLAASSEN, C. D.: High-performance liquid chromatographic analysis of glutathione and its thiol and disulfide degradation products. J. Chromatogr. **318:** 259–270, 1986.

SZEWCZUK, A., MILNEROWITZ, H., POLOSATOV, M. V. AND SOBIECH, K. A.: Immunofluorescent localization of γ-glutamyl transferase in rat and bovine tissues. Acta Histochem. **66:** 152–159, 1980.

TATE, S. S. AND MEISTER, A.: Interaction of γ-glutamyl transpeptidase with amino acids, dipeptides, and derivatives and analogs of glutathione. J. Biol. Chem. **249:** 7593–7602, 1974.

WICKBERG, E.: Isolation of a mixed disulfide of glutathione and cysteinylglycine from a partial hydrolysate of glutathione. Nature (Lond.) **172:** 398, 1953.

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