Relationship between glutathione content in liver and glutathione conjugation rate in the rat *in vivo*

Effect of buthionine sulfoximine pretreatment on conjugation of the two 2-bromoisovalerylurea enantiomers during intravenous infusion

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The relationship between hepatic glutathione content and hepatic glutathione conjugation rate in the rat *in vivo* was investigated. As substrate for glutathione conjugation, racemic (*R,S*)-2-bromoisovalerylurea (BIU) was used which gives rise to the biliary excretion of two diastereoisomeric glutathione conjugates and the urinary excretion of two diastereoisomeric mercapturates. The excretion rate of the glutathione conjugate in bile reflects hepatic conjugation exclusively. An intravenous infusion of BIU was given and the excretion rates of the metabolites in bile and urine were determined. The glutathione concentration in the liver was followed by taking biopsies every hour. Glutathione was depleted by the infused substrate; in rats that were pretreated with the inhibitor of glutathione biosynthesis, buthionine sulfoximine (BSO), the depletion of the glutathione content was more rapid. The rate of excretion of the glutathione conjugate in bile was plotted against hepatic glutathione content. These results indicate that the 'organ K_m ' for glutathione in the liver is approximately 0.5 $\mu\text{mol/g}$ of liver, so that the hepatic glutathione conjugation rate is decreased only at severe glutathione depletion.

INTRODUCTION

Glutathione (GSH) conjugation is an important biotransformation reaction of xenobiotics and several endogenous compounds. In most cases it forms much less toxic products, but for some classes of compounds the conjugates are more toxic than the parent compound [1,2]. Therefore, GSH conjugation plays a decisive role in toxification and detoxification of many xenobiotics. Obviously the efficiency of this biotransformation depends on the availability of the co-substrate for conjugation, the tripeptide GSH. Although it is present in many tissues at a high concentration (for instance, in the liver at 5 $\mu\text{mol/g}$ of liver), it can readily be exhausted at high doses of substrates for conjugation. Thus, depletion of GSH is usually achieved by administration of diethyl maleate (DEM) or phorone [3], which are both very good substrates for GSH conjugation. Many hepatotoxins similarly cause GSH depletion in the liver [4].

At present little is known about the relationship between the tissue GSH concentration and GSH conjugation rate in that tissue, either *in vivo* or in a perfused organ. The *in vitro* K_m -values of the purified glutathione transferases towards GSH are of the order of 0.05–0.3 mM in most reports [5–9]. If a tissue content of 5 $\mu\text{mol GSH/g}$ is 'translated' into a 10 mM concentration in the cell cytosol [10], and if the transferases had a K_m -value of 0.3 mM towards GSH, this means that the rate of GSH conjugation in such a cell would be relatively little affected by even an 80% decrease in cellular GSH. This may have implications for studies in which the role of GSH in the toxicity of a compound is evaluated by a pretreatment which decreases GSH to low levels: the question is whether these levels are low enough seriously to impair the rate of GSH conjugation. Of course, when GSH is totally depleted the conjugation becomes impossible.

In this paper we have evaluated the GSH conjugation rate in rat *in vivo* in relation to hepatic GSH availability. We have used racemic (*R,S*)-BIU as substrate, which is rather non-toxic so that it can be infused intravenously at very high doses without generating any apparent toxic effect. Two diastereoisomeric GSH conjugates are formed from the two enantiomers of this substrate that are excreted in bile, while the two diastereoisomeric mercapturates formed by breakdown of the GSH conjugates are excreted only in urine. The GSH conjugates excreted in bile reflect hepatic synthesis exclusively [11–15]. The mercapturates of the two enantiomers excreted in urine represent both hepatic and possible extrahepatic GSH conjugation. The results indicate that GSH conjugation in the liver has a low 'apparent K_m ' for GSH, so that only depletion below 1.0 $\mu\text{mol/g}$ of liver will seriously affect the GSH conjugation rate.

MATERIALS AND METHODS

Materials

Racemic BIU was obtained from the Onderlinge Pharmaceutische Groothandel (OPG), Utrecht, The Netherlands (Pharmacopoeia quality). Buthionine sulfoximine (BSO) and bovine serum albumin (BSA) (Fraction V) were from Sigma, St. Louis, MO, U.S.A. All other chemicals were from sources as used before [16].

Rats

Male Wistar rats that had free access to food and water were obtained from the Sylvius Laboratory animal rooms. Body weight was between 215 and 235 g. They were anaesthetized with pentobarbital (Nembutal®) by intraperitoneal injection and kept under anaesthesia during the experiments (6–7 h) by repeated

Abbreviations used: BIU, 2-bromoisovalerylurea; BSO, buthionine sulfoximine; DEM, diethyl maleate; IU-MA, isovalerylurea mercapturate; NPT, non-protein thiols.

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administration of small doses of pentobarbital. The bile duct, urine bladder and external jugular vein were catheterized as described elsewhere [17]. An infusion of D-mannitol, preceded by a bolus injection to reach steady state more rapidly, was administered *via* the jugular vein to achieve a constant urine flow [17]. The rats were maintained at 37.5–38.5 °C by keeping them on a heating pad. During the 30 min after surgery, blank urine and bile were collected. The experiment was started by administration of a bolus injection of 10 μ mol of BIU (0.1 ml of a 0.10 M-BIU solution in dimethyl sulphoxide), followed by continuous infusion of 55 μ mol BIU/h (in a solution containing 10 mM-BIU, 20 mg of BSA/ml and 75 mg of D-mannitol/ml; infusion rate 5.5 ml/h). This infusion was continued for 6–7 h. Bile and urine were collected in 15-min fractions. The rats pretreated with BSO received an intravenous injection of 1 ml of 0.45 M-BSO in saline 1 h before the BIU infusion started. Liver biopsies were taken every hour by cutting 10 mg (first three hours) to 100 mg samples (later hours) from the edge of the front lobes; they were immediately put in liquid nitrogen for later analysis. The bleeding was stopped by putting a piece of Willispon (a gelatin-based styptic material; Willpharma, Zwaneburg, the Netherlands) against the cut edge. The volume of the bile, urine and liver samples was determined by weighing.

Assays

The GSH conjugates and mercapturates of BIU were assayed as described previously [16]. Briefly, this includes separation of the diastereoisomeric conjugates by h.p.l.c., followed by electrochemical detection of the compounds. For this technique no work-up of the bile and urine samples other than dilution is required.

Glutathione in the liver samples was determined as non-protein thiols using Ellman's reagent [18,19]. To the liver sample was added 550 μ l of 5% (w/v) 5-sulphosalicylic acid, followed by homogenization (on ice). To 0.5 ml of the supernatant obtained after centrifugation was added 1.0 ml of 0.5 M-Tris/HCl buffer (pH 9.0) containing 20 mM-EDTA. The absorbance of a portion (1300 μ l) of this mixture in the cuvette of the spectrophotometer was read at 412 nm, after which 50 μ l of 10 mM-5,5'-dithiobis(2-nitrobenzoic acid) in 0.1 M-sodium phosphate buffer (pH 7.0) containing 20 mM-EDTA was added, followed by mixing. After 90 s the absorbance at 412 nm was again read. The non-protein thiol concentration was calculated by using a standard curve for GSH made at the same time as the assay of the liver samples.

RESULTS

Biliary and urinary excretion of GSH conjugates and mercapturates after BIU infusion in relation to hepatic GSH content

In a first series of experiments racemic (*R,S*)-BIU was infused at a high rate, preceded by a bolus dose in order to deplete GSH (detected as non-protein thiols) more rapidly. Over the initial 4 h of the infusion the hepatic GSH content decreased steadily (Fig. 1), until a low constant level was reached. During the first 1 h of the experiment, a rapid increase in the biliary excretion of the two diastereoisomeric GSH conjugates was observed, followed, after the second hour, by a steady decline of the excretion rate (Fig. 2). This was the same for both diastereoisomeric conjugates, except that the excretion rate for the GSH conjugate derived from the (*R*)-BIU enantiomer was somewhat higher than that for the (*S*)-BIU enantiomer. The urinary excretion of the mercapturates (IU-MA) (derived from the GSH conjugates by breakdown in the kidneys, followed by *N*-acetylation) showed a different time course: the rate increased continuously for (*S*)-IU-

MA, formed from (*R*)-BIU, while that for (*R*)-IU-MA became more or less constant after 3 h, but at only one-third of the rate of its diastereoisomer (Fig. 3).

Effect of BSO

When rats were pretreated with BSO, an inhibitor of GSH synthesis [20], the initial GSH concentration was lower, but upon BIU infusion it declined at about the same rate as in the control rats (Fig. 1). After 3 h, hepatic GSH was virtually exhausted. The result of this can be seen in Fig. 2: the biliary excretion of the GSH conjugates virtually completely stopped after 3 h. Similarly, the urinary excretion of (*S*)-IU-MA decreased after 3 h, but that of (*R*)-IU-MA remained more or less constant (Fig. 3).

Relationship between hepatic GSH content and biliary excretion rate of the GSH conjugates

In order to evaluate the relationship between hepatic GSH content and the rate of hepatic GSH conjugation we plotted the GSH content against the biliary excretion rate of the sum of the diastereoisomeric GSH conjugates excreted during the 15 min after the time-point of sampling for the GSH assay (Fig. 4). The

Fig. 1. Hepatic GSH content in rats infused with BIU, and effect of BSO pretreatment

□, Controls; ■, BSO-treated. Results given are mean $\pm$ S.E.M.

Fig. 2. Rate of biliary excretion of the GSH conjugate

Conjugate derived from (*R*)-BIU (a) and (*S*)-BIU (b) in control (□; top) and BSO-treated (■; bottom) rats. Values are means $\pm$ S.E.M.

Fig. 3. Rate of urinary excretion of mercapturates

Mercapturates were derived from (*R*)-BIU (a) and (*S*)-BIU (b) in control (□; top) and BSO-treated (■; bottom) rats.

Fig. 5. Sum of the biliary GSH conjugate excretion and the urinary mercapturate excretion plotted against time of infusion

(a) Data for the derivatives of (*S*)-BIU; (b), those for (*R*)-BIU. □, Controls (top); ■, BSO-treated (bottom).

Fig. 4. Combined rate of biliary excretion of the two diastereoisomeric GSH conjugates plotted against hepatic GSH content

The hepatic GSH content at each hour was plotted against the GSH conjugate excretion in bile during the following 15 min for each rat separately. □, Controls; ■ and ▲, BSO-treated (see text).

zero-time GSH content was not used in this analysis, since only then had the BIU infusion started. Since the two diastereoisomeric GSH conjugates showed virtually identical dependence on GSH content, we have used the sum of the two conjugates. The 15-min period after the GSH assay point was chosen because the biliary excretion is very rapid after hepatic synthesis [14], and this time period will therefore most likely be the best to reflect the hepatic conjugation rate at that particular GSH content. For this plot we also used an additional three rats which were pretreated with BSO and received a BIU infusion almost identical with that of the rats used in generating the data in Figs. 2 and 3. The rates for the rats pretreated with BSO and the controls overlap so that there does not seem to be a different GSH-dependence for the two groups.

DISCUSSION

It is difficult to assess the *in vivo* GSH conjugation rate of a substrate in relation to the availability of GSH in an organ: continuous monitoring of GSH in an organ may be impossible

because it is not easy to take biopsies continuously, and it may be impossible to follow *in situ* the formation of the conjugate in that organ selectively. In this study we have made use of the fact that liver biopsies can be easily taken, and that BIU is excreted in bile only as the GSH conjugate when it is synthesized inside the liver [15]. Moreover, the biliary excretion of hepatic conjugates is rapid and, for the (*R*)- and (*S*)-BIU enantiomers, reflects their rate of conjugation [14]. We have assumed that the biliary excretion rate is not limiting. After 75 min (Fig. 2) the rate of biliary excretion starts to decrease, so that only the first data point that has been used for the analysis in Fig. 4 could eventually have been affected if biliary excretion were rate limiting during those 75 min. The different time courses for biliary excretion of the GSH conjugates for the control and BSO-treated rats already indicate that, certainly in the BSO-treated rats, rate-limiting biliary excretion is very unlikely. Therefore, the present results allow us to probe the relationship between GSH content in the liver *in situ* and hepatic GSH conjugation rate. In Fig. 4 the hepatic GSH content has been plotted against the rate of biliary excretion of the sum of the two conjugates. A half-maximum velocity of GSH conjugation is observed at approximately $0.75 \mu\text{mol}$ of non-protein thiols (NPT)/g of liver. Part of this NPT level will represent low-molecular-mass thiols other than GSH, or GSH in the mitochondrial pool which may not be available for conjugation, so that the actual, very 'apparent K_m ' for GSH may be of the order of $0.25\text{--}0.5 \mu\text{mol/g}$ of liver. Assuming that this GSH is homogeneously distributed in all liver cells, and that it is contained in the cytosol only (see [10]), the apparent K_m -value in cytosol would be of the order of 0.5 mM .

A number of assumptions have been made in this analysis. The first relates to the fact that the GSH conjugates formed in the liver may be excreted both into bile and into blood; in the latter case it ends as mercapturate in urine. The assumption is that the distribution between biliary and blood excretion of the GSH conjugates formed in the liver does not change as a function of the rate of conjugate formation inside the liver. There are no data against such a concept, but there is no proof for it either. In the second place we have assumed equilibrium between conjugation

and biliary excretion after 60 min: for Fig. 4 we have used only the results obtained after 60 min. If the biliary and urinary excretion of the GSH conjugate and mercapturate for each BIU enantiomer are added together (Fig. 5) the results indeed indicate that the total rate of GSH conjugate plus mercapturate excretion for the BIU enantiomers reaches an apparent steady state after 90 min in the control rats. The fact that urinary excretion of the mercapturates increases continuously (while biliary elimination decreases) suggests that extrahepatic conjugation may take over from hepatic conjugation as hepatic GSH decreases. Previous work has shown that the urinary excretion is much 'slower' than biliary excretion [11]. Therefore, the steady state in biliary excretion would certainly be reached long before those 90 min. However, at the same time it has to be realized that, due to the aim of this study, it is by definition impossible to reach the steady state properly: because we want to study GSH dependence we have to achieve a decrease in GSH availability, which unavoidably changes the system continuously as GSH is increasingly depleted. This is also indicated by the fact that urinary excretion of unchanged BIU increases continuously during the experiment (M. Polhuijs, G. Lankhaar and G. J. Mulder, unpublished work): as clearance capacity (i.e., GSH conjugation) is lost due to decreased availability of GSH, BIU accumulates to higher levels. The assumption that biliary excretion rate is not limiting has already been discussed above; since data on the intracellular level of the GSH conjugates are not available we cannot prove that there is a linear relationship between intracellular conjugate level and biliary excretion rate. Eventual accumulation of the GSH conjugates might also lead to product inhibition of GSH conjugation [21]; however, we have no indication that it plays a role for the BIU conjugates.

It would be incorrect to correlate total excretion data (urine + bile) with hepatic GSH levels. The data of Fig. 5 indicate that, after BSO treatment, the conjugation rate in the whole body drops, mainly because of the loss of hepatic conjugation as reflected in the decreased biliary excretion of the GSH conjugates. However, we do not know as yet whether extrahepatic conjugation plays an important role in GSH conjugation of the BIU enantiomers. Moreover, the time course for biliary and urinary excretion of the conjugates is very different for a number of (pharmacokinetic) reasons [11,14,15] so that one cannot compare the hepatic GSH content at a certain time with the urinary excretion rate immediately thereafter. There is a much longer delay between synthesis and urinary excretion than there is between hepatic synthesis and biliary excretion.

In spite of the above considerations, the present results allow an estimate of the *in vivo* apparent K_m for GSH in the liver towards GSH conjugation to be made. The results indicate that the GSH concentration may drop considerably before the rate of conjugation becomes affected: a drop of hepatic GSH content

from 5 to 1 $\mu\text{mol/g}$ of liver will reduce the conjugation rate by only some 20%. This shows that GSH availability rather than concentration *per se* is decisive: even at low GSH concentration the conjugation rate is appreciable, but conjugation becomes impossible when no new GSH can be synthesized. Whether the same applies to other organs cannot so easily be studied, since the essential tool of the rapid biliary excretion is not available in other organs. However, the fact that the organ K_m for GSH in the liver is similar to the K_m -value for the isolated transferases suggests that a similar organ K_m may apply for other organs as well.

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