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DRUG-INDUCED LIPID PEROXIDATION IN MICE—III

GLUTATHIONE CONTENT OF LIVER, KIDNEY AND SPLEEN AFTER INTRAVENOUS ADMINISTRATION OF FREE AND LIPOSOMALLY ENTRAPPED GLUTATHIONE

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Abstract—The half-life of extracellular glutathione was found to be 1.9 min in fed mice with a hepatic glutathione content of 44 ± 10 nmol glutathione per mg protein. It was 4.9 min in animals that had been fed for 48 hr a liquid sucrose diet resulting in a decreased hepatic glutathione of 25 ± 7 nmol/mg. A single intravenous injection of 16.2 μmol liposomally entrapped glutathione led to an increase in hepatic glutathione to 45 nmol/mg in the sucrose-fed mice after 2 hr and had no effect in the fed group. The spleen glutathione content reached a maximum at 30 min after injection in both groups. The maximum uptake into liver was 21% of the applied dose, into the spleen 7% and into the kidneys 2.4%. Injection of glutathione in solution led to a similar increase of hepatic glutathione as observed with GSH-containing liposomes, while liposomes filled with the constituent amino acids had only a marginal effect. The spleen took up only liposomal GSH. In contrast, the kidney glutathione content increased within 10 min up to 150% upon injection of free glutathione. The findings are consistent with a rapid hydrolysis of extracellular free glutathione followed by an interorgan turnover utilizing the constituent amino acids for resynthesis in the liver. Pretreatment of the animals with the glutathione synthesis inhibitor buthionine sulfoximine essentially abolished the hepatic glutathione increase upon treatment with GSH-liposomes or with the free compound. The finding that only liposomally entrapped glutathione protects mice against liver necrosis induced by highly dosed paracetamol is discussed with respect to differential uptake and distribution of GSH-liposomes in the liver.

The most important intracellular non-protein thiol, the tripeptide glutathione, is synthesised within mammalian cells by two consecutive enzyme-catalysed cytosolic reactions requiring ATP. It would appear that the availability of the precursor amino acids cysteine [1] and methionine [2] is the factor that limits the overall biosynthesis of GSH in the liver. On the other hand, liver cells release oxidised and reduced glutathione as well as glutathione conjugates [2, 3] which are subject to rapid degradation extracellularly. Renal brush border γ-glutamyltranspeptidase had been recognised as a major site of this degradation indicating that there is an interorgan turnover of glutathione [4–6]. The impermeability of liver cells [4] to extracellular glutathione in its form as a tripeptide and the effectiveness of extracellular degradation imply a very low extracellular steady state concentration of glutathione, which in arterial rat plasma is 3 μmol/l [7] and in human venous plasma 0.3 μmol/l [8]. In man, a half-life of 1.6 min was determined for extracellular glutathione [8]. In rats, a distinct diurnal variation was observed in the hepatic glutathione content [9, 10]. Also starvation of mice was shown to decrease the liver glutathione to approximately 60%, accompanied by a enhanced susceptibility to drug toxicity [11, 12]. In addition to these variations in time, a histochemical study showed that within the rat liver a decreasing GSH gradient exists from the periportal to the centrolobular region [13]. These observations suggest that the assessment of the biological potency of glutathione has to take into account numerous factors including diurnal variations, topology within organs and nutritional status.

In an accompanying paper, we demonstrated protection of mice pretreated with GSH-filled liposomes against paracetamol-induced liver necrosis [14]. In this study, as well as in a previous one by others [15], a limited but significant protection was also observed after intravenous injection of free glutathione. Therefore, we studied the pharmacokinetics of these different forms of intravenously administered glutathione in mice with respect to uptake into liver and other organs.

MATERIALS AND METHODS

Male albino mice were kept on a commercial diet for at least three weeks (Altromin, Lage, W. Germany, diet C 1018) as described [12]. Some of the animals were fed a liquid 10% (w/v) sucrose diet 48 hr prior to the experiments; these animals are designated "sucrose-fed". All animals were pretreated by benzo(*a*)pyrene (3 × 20 mg/kg i.p.) as described [12]. All experiments were started at 5–9 p.m. Glutathione was injected into the tail vein without immobilisation or anesthesia of the animals; for short time determinations (≤30 min), the solution was injected in the vena cava inferior after anesthesia with Nembutal® (90 mg/kg). For the time

course studies both types of injections were used (overlapping at 30 min). Blood was withdrawn by heart puncture, immediately centrifuged for 1 min at 13,000 *g*; 0.1 vol of 300 g/l metaphosphoric acid with 1 mmol/l ethylenediaminetetraacetic acid were added to serum; precipitated protein was removed by centrifugation and glutathione was determined [16].

After the experiments, the animals were killed by cervical dislocation and their livers immediately perfused for 30 sec with ice-cold saline via the left ventricle while the upper part of the vena cava inferior was opened and the lower part was ligated. The organs were immediately frozen in liquid N_2. Frozen sections were homogenised in 3% metaphosphoric acid and glutathione was determined according to method I described in Ref. [16]. D,L-Buthionine-S,R-sulfoximine was synthesised and purified according to Ref. [17]. Benzo(*a*)pyrene was purchased from Sigma Chemicals, Nembutal® from Abbott, reduced and oxidised glutathione were a gift from Boehringer, Mannheim. Large univesicular neutral liposomes were also obtained from Boehringer, Mannheim, W. Germany. They contained 25 mg GSH/ml in 5 mmol/l potassium phosphate buffer (pH = 6.3) including 0.15 mol/l NaCl [14]. "Empty" control liposomes contained only isotonic saline or equimolar amounts of glutamate, cysteine and glycine ("amino acid liposomes"). Liposomes or glutathione solutions (freshly prepared in the same buffer) were always injected in the same volume of 0.2 ml within 10 sec.

RESULTS

The hepatic content of total free glutathione in mice exhibits a diurnal rhythm with a maximum at 10–12 a.m. (63 ± 14.6 nmol/mg protein, $n = 7$) and a minimum between 6 and 8 p.m. (44.1 ± 10.2 nmol/mg, $n = 6$). If the animals were fed a protein-free diet for 48 hr, e.g. liquid sucrose, an essentially, constant glutathione content of 25 ± 7.4 nmol/mg, $n = 10$ was measured. This might be due to a constant low availability of sulfur amino acids [18]. First of all, we determined the apparent half-life of a single loading dose of intravenous free GSH in mouse plasma. Figure 1 illustrates that for fed animals, a $t_{\frac{1}{2}} = 1.9$ min is obtained, whereas $t_{\frac{1}{2}} = 4.9$ min is determined for sucrose-fed mice. If we assess the total interstitial (i.e. extracellular) volume as about 20% of the animals body weight, without clearance an equilibrium concentration of ~3 mmol/l is calculated for a hydrophilic substance after complete distribution into this space. The extrapolation of the half-logarithmic straight line to zero time gives a similar value indicating that a rapid complete distribution has taken place within the first minute. Hence, the elimination rate for glutathione appears to be faster in fed (high liver glutathione) mice compared to sucrose-fed (low liver glutathione) animals. In order to find a rationale for the observed strongly protective effect of liposomal GSH against paracetamol-induced liver necrosis reported in the preceding paper, we studied the time course of the uptake of liposomally entrapped GSH into various organs and its dependence on the nutritional state of the animals. The upper panel of Fig. 2 shows that within 2 hr the liver of sucrose-fed mice doubles its glutathione content following a single injection of liposomally encapsulated GSH. The applied dose was 16.2 μmol GSH per animal (0.55 mmol/kg), the

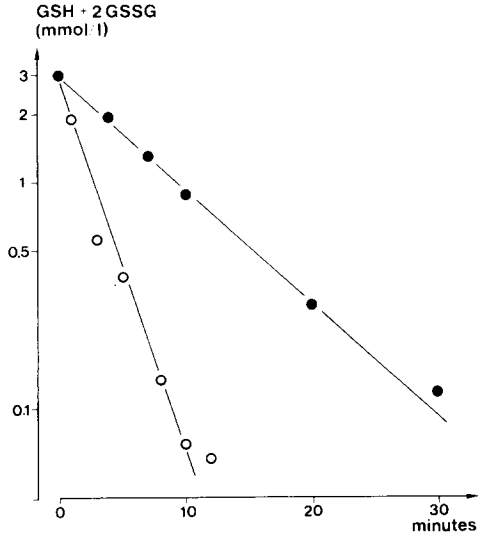

Fig. 1. Apparent half-life of plasma glutathione in fed (○) and 48 hr sucrose-fed (●) mice which received a single intravenous dose of 16.2 μmol GSH. The mean obtained with 2–5 animals is plotted. The correlation coefficients with the whole set of data were $r = 0.977$ (fed) and $r = 0.946$ (sucrose-fed).

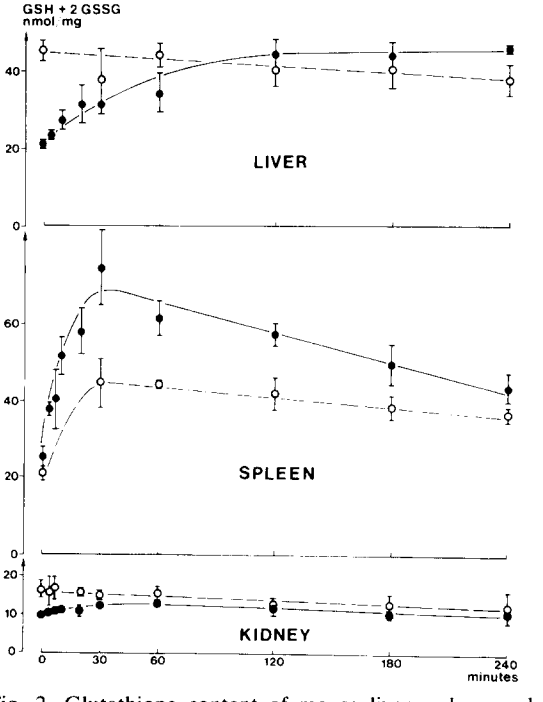

Fig. 2. Glutathione content of mouse liver, spleen and kidney of fed (○) and 48 hr sucrose-fed (●) mice, after a single intravenous injection of 16.2 μmol liposomally entrapped GSH. Data are mean values ± S.D. obtained with 3–6 animals and expressed in GSH equivalents per mg of protein.

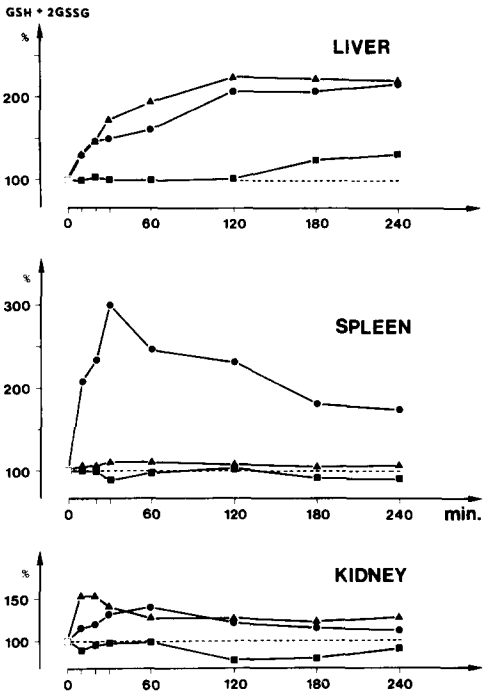

Fig. 3. Glutathione content of mouse organs after single intravenous injections of equivalent doses of 0.55 mmol/kg of free GSH (▲), liposomally entrapped (●) and liposomes filled with an equimolar mixture of glutamate, cysteine and glycine (■). (Data plotted as means from 3 to 4 mice, minimal S.D. = 1.2%, maximal = 20%.) 100% corresponds to the 0 time value for sucrose-fed animals in Fig. 2.

hepatic uptake equals 3.6 μmol (calculated from the specific glutathione content on a constant protein basis of 185 mg protein per gram liver wet weight, mean organ weight 1 g). In contrast, the livers of the fed group showed no further increase of its liver GSH content within this time. Obviously, an upper limit of ~45 nmol/mg (i.e. a concentration of 8 mmol/l) is not exceeded. As expected for an organ of the reticuloendothelial system, the spleen takes up considerable amounts of GSH compared to its content. Again, a pronounced influence of the nutritional state is observed (Fig. 2, middle panel). The spleens of both groups reach a maximum glutathione content half an hour after injection of the liposomes. In the fed group, this maximum corresponds to an increase by 1.1–1.2 μmol GSH, i.e. 7% of the applied load. Finally, the lower panel in Fig. 2 demonstrates that the renal uptake of liposomal GSH (~0.4 μmol = 2.4%) is negligible within the time under consideration.

It appears that the liver takes up extracellular glutathione only under conditions, where a certain maximum amount is not yet reached. Therefore, we compared the apparent uptake of free GSH, liposomally entrapped GSH and an equivalent amount of liposomally encapsulated constituent amino acids into the organs of sucrose-fed mice. Figure 3 shows in the upper panel that within 2 hr the livers of these animals incorporate a similar amount of glutathione from an injection of the free compound as from the entrapped compound; the time course for the "uptake" from free glutathione seems to be marginally faster. With the amino acid-filled liposomes, a small and delayed increase in hepatic glutathione is observed. The middle panel shows that the spleen does not incorporate GSH but from liposomes. Presuming the phagocytosis of intact liposomes, the organ seems not to be able to utilise the encapsulated constituent amino acids of GSH for the synthesis of the tripeptide. This holds true for the kidney in agreement with the view that liposomes are not filtered by the glomerulus. However, administration of free GSH leads to an initial increase in renal glutathione within the first 20 min. The slow continuous decrease of the renal content is obviously paralleled by the hepatic increase suggesting a shift of renally metabolised products to the liver and its reutilisation by this organ.

The experiments performed so far do not allow differentiation between uptake of the intact tripeptide or an extracellular hydrolysis followed by amino acid uptake and intracellular resynthesis. Therefore, we used an *in vivo* inhibitor of glutathione biosynthesis. The most potent and specific agent of this class of compound seems to be buthionine sulfoximine (BSO) [17]. With this respect, the data in Table 1 confirm previous work [24] by showing a maximum decrease of hepatic GSH by 80% 4 hr after BSO administration. The splenic GSH is also affected; however, the effect is much less pronounced. It

Table 1. Effect of *in vivo* inhibition of glutathione biosynthesis by pretreatment with 4 mmol/kg buthionine sulfoximine (BSO) on the hepatic and splenic uptake of glutathione from intravenously administered free GSH or GSH-filled liposomes. Data are given as means ± S.D. in nmol GSH equivalents per mg protein; n = 3–4; 0 = time of intravenous injection of the animals with 0.55 mmol/kg glutathione; BSO was given 2 hr prior to the injections

	Time (hr)	BSO	GSH	BSO + GSH	GSH-liposomes	BSO + GSH-liposomes
Liver glutathione	−2	29.7 ± 2.1	—	—	—	—
	0	11.5 ± 2.5	32.0 ± 0.8	11.4 ± 0.2	32.0 ± 0.8	11.4 ± 0.2
	+2	6.4 ± 1.2	51.3 ± 3.5	12.4 ± 0.4	50.8 ± 4.6	12.1 ± 1.8
	+4	8.8 ± 0.9	45.6 ± 5.2	14.9 ± 1.0	51.2 ± 6.1	15.8 ± 0.4
Spleen glutathione	−2	25.0 ± 3.5	—	—	—	—
	0	25.1 ± 3.0	27.0 ± 1.6	26.2 ± 1.5	27.0 ± 1.6	26.2 ± 1.5
	+2	21.6 ± 3.1	30.6 ± 1.8	19.5 ± 1.5	59.4 ± 1.6	40.1 ± 6.7
	+4	16.9 ± 2.9	29.3 ± 0.4	18.5 ± 0.2	39.8 ± 2.2	28.0 ± 3.1

seems that in liver the treatment with GSH or GSH-liposomes prevents a further decrease of hepatic GSH beyond the reduced content which had been reached 2 hr after BSO injection, i.e. at the time of GSH administration. In spleen, the GSH treatment does not significantly change the BSO-induced slight GSH decrease, while the liposome treatment leads still to a drastic, albeit smaller increase in the GSH content of the organ. The prominent point, however, is that the GSH- or GSH-liposome-induced hepatic GSH increase is completely abolished after BSO pretreatment. If one does not infer that BSO inhibits the hepatic uptake of liposomes these data force the conclusion that also liposomally incorporated hepatic glutathione stems from resynthesis. In contrast, the experiment using amino acid-filled liposomes (Fig. 3) suggests that the liver does only minimally utilize the constituents imported via liposomes for the resynthesis of the tripeptide.

DISCUSSION

This study shows first of all that in agreement with the data obtained for man [8] the half-life of exogeneous glutathione in the circulation of the mouse is very short. The observation that this half-life appears to be prolonged roughly four times in animals with an alimentary lowered hepatic GSH may be interpreted as a mechanism suitable to economize GSH by a reduced turnover. In spite of this short half-life of GSH in plasma, intravenous administration of free GSH leads in sucrose-fed mice to a two-fold increase of hepatic GSH after 2 hr lending further support to the view [4] that after renal degradation an intraorganal shift of precursor amino acids from the kidney to the liver enables hepatic resynthesis of glutathione (cf. time course of hepatic and renal GSH in Fig. 3).

The further experiments of this work, however, show that no straightforward correlation between the pharmacokinetics of glutathione and its physiological effect in the liver seems to exist. With liposomes for which a half-life of 2 hr has been described in mice [19], we observe a 100% increase of hepatic GSH within 2 hr only in animals with an already alimentary lowered hepatic glutathione content. A similar time course is found in animals treated with GSH solution. The biological response of these two groups of mice with practically identical hepatic GSH, however, is strikingly different: the GSH-liposome treated mice are nearly totally resistant to drug-induced liver necrosis, whereas the GSH solution treated animals are much less protected [14]. This means that the pharmacological effect does not depend on the total amount of drug, i.e. glutathione delivered to the target organ, but is dependent on the form of administration. The types of experiments presented here do not allow to examine the different possible interactions of liposomes with cells, such as stable adsorption, endocytosis, fusion and lipid transfer [20–22]. The preferential localization of Kupffer cells around the portal vein, however, and the 0.1 μm pore size of the endothelial sieve plate in the liver seems to justify the assumption that the liposomes are initially phagocytised by these cells. This does not exclude transitory interactions with or adhesion to other sinusoidal cells but rules out a direct initial contact with parenchymal cells. On the other hand, the biological response of the liver of treated animals, the resistance to centrolobular necrosis, reflects a parenchymal cell function. The analysis of the bile conjugates of the drug indicates that drug metabolism itself was not impaired in the liposomes-treated animals [14], i.e. the apparent protection was not caused by inhibition of monooxygenase system. Consequently, the non-equivalence of liposomal GSH and free GSH could be due to a difference of GSH distribution in the liver, i.e. a mostly sophisticated interaction of different cell populations of largely unknown metabolic equipment must exist. The histochemically observed oppositely directed hepatic gradients of glutathione [13] and cytochrome P-450 [23], the different elimination kinetics of parecetamol after normal or retrograde liver perfusion in the rat [25], as well as the totally different enzymatic profile of parenchymal and non-parenchymal liver cells [26] lend support to this view derived here from *in vivo* observable functional differences.

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