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GLUTATHIONE ENHANCEMENT IN VARIOUS MOUSE ORGANS AND PROTECTION BY GLUTATHIONE *iso*PROPYL ESTER AGAINST LIVER INJURY

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Abstract—Intraperitoneal administration of glutathione *iso*propyl ester to fasted, male NMRI mice dose dependently increased the glutathione concentration in various organs. Administration of 1 g/kg glutathione *iso*propyl ester led to the following increases: liver 166%; lung 164%; heart 121% after 4 hr; and brain 133% after 6 hr. Spleen, kidney, muscle, serum and blood cell glutathione were not affected by the treatment. Pretreatment with glutathione *iso*propyl ester was found to protect against paracetamol- or allyl alcohol-induced liver damage. Following treatment with the ester a significant correlation between protection against liver damage and enhancement of liver glutathione content was obtained. The dose dependence of this protection was studied.

The important role of glutathione (GSH†) for many cellular functions is established biochemical knowledge [1, 2]. At present, more than 20 glutathione dependent enzymes are known. Beyond its function in cellular defense, glutathione has been implicated in such diverse phenomena as metabolic regulation via thiol-disulfide exchange [3], multi drug resistance [4], leukotriene metabolism [5], lymphocyte function [6], protein and DNA synthesis [7].

As a consequence of this increasing knowledge on the pivotal role of GSH means have been sought to manipulate the glutathione status *in vivo* as well as *in vitro*. Since the tripeptide is strongly hydrophilic it does not readily diffuse into cells. Nevertheless, i.v.-application of glutathione to animals leads to an elevation of intracellular glutathione in organs such as the liver; but this elevation is most likely due to extracellular breakdown of GSH, uptake of the fragments and intracellular resynthesis of the tripeptide [8]. L-2-Oxothiazolidine-4-carboxylic acid (ATCA), a compound which is metabolized to a glutathione precursor, is an alternative means for increasing intracellular glutathione in experimental animals. However, neither glutathione nor ATCA when given i.v. were protective against e.g. paracetamol-induced liver injury, a chemical insult which results in severe glutathione depletion [9].

An alternative promising way of raising cellular glutathione has been devised by Meister who suggested the use of glutathione esters [10]. The idea is that glutathione esters are hydrophobic enough to diffuse into cells, where they supposedly are cleaved by unspecific esterases, thus ultimately carrying free glutathione into the cell. The validity of this concept has already been demonstrated by showing that application of glutlathione ethyl ester led to transient increases of GSH in various mouse organs [11]. Furthermore, administration of glutathione methyl ester was protective against acetaminophen-induced lethality [10]. A protective effect of glutathione *iso*propyl ester against cadmium-induced kidney damage was also reported [12]. Therefore it appears that glutathione esters may be useful as therapeutic agents.

This study was designed in order to establish the pharmacokinetics of glutathione *iso*propyl ester. Furthermore, we were interested in applying the result of the pharmacokinetic properties of glutathione *iso*propyl ester to the question whether appropriate administration of this compound would protect mice against the hepatotoxic effects of two well studied hepatotoxins, i.e. allyl alcohol and acetaminophen.

MATERIALS AND METHODS

Male NMRI albino mice were purchased from Thomae, (Biberach, F.R.G.). They were kept at least 1 week at 27° and 55% relative humidity in a 12 hr day/night rhythm at 100 lux illumination on a standard diet (Altromin, No. 1314) with free access to food and water. Animals were starved 24 hr before the experiment.

N-(*N*-L-Glutamyl-1-cysteinyl)glycine L-*iso*propyl ester sulfate monohydrate (YM737) was a gift from Yamanouchi Ltd (Japan). Glutathione *iso*propyl ester was dissolved in water and adjusted to pH 7 immediately before application (i.p.). Animals treated with allyl alcohol (75 μL/kg, i.p.) or acetaminophen (400 mg/kg) were kept on a 10%-sucrose diet (w/v) for 48 hr prior to the experiment. Animals which received acetaminophen were additionally pretreated with benzo[*a*]pyrene (3 × 20 mg/kg i.p.) as described in Ref. 13.

After the experiment the animals were killed by cervical dislocation. Blood was withdrawn by heart puncture into 2.5% heparin and centrifuged. Blood cells were washed with 0.9% NaCl prior to lysis with

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† Abbreviations: GSH, glutathione; GOT, serum aspartate amino transferase (EC 2.6.1.2); GPT, serum alanine amino transferase (EC 2.6.1.1); i.p., intraperitoneal; i.v., intravenous; SDH, sorbitol dehydrogenase (EC 1.1.1.14).

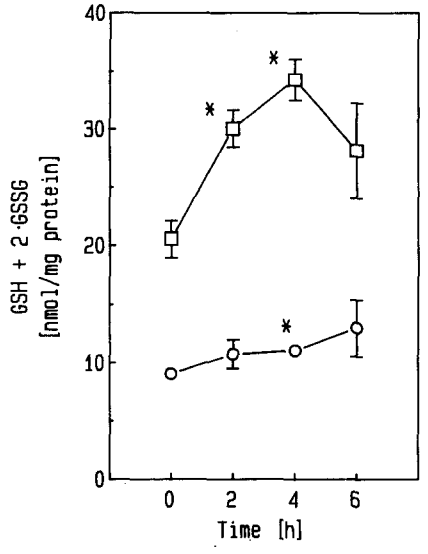

Fig. 1. Time dependence of the concentration of total glutathione in liver (□) and heart (○) of starved mice following intraperitoneal administration of 1 g/kg glutathione *iso*propylester (data ± SE, * $P < 0.05$, *t*-test, N = 3).

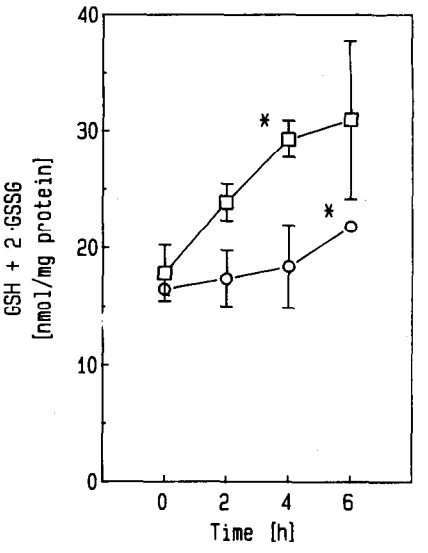

Fig. 2. Time dependence of the concentration of total glutathione in lung (□) and brain (○) of starved mice following intraperitoneal administration of 1 g/kg glutathione *iso*propylester (data ± SE, * $P < 0.05$, *t*-test, N = 3).

0.1 M HCl. Liver injury was assessed by measurement of serum alanine aminotransferase (SGPT), serum aspartate aminotransferase (SGOT) and sorbitol dehydrogenase (SDH) activities. The organs were perfused with cold 0.9% NaCl and immediately frozen in liquid N_2. Frozen sections were homogenized in 3% metaphosphoric acid to give a 20% homogenate. The homogenates were centrifuged and the supernatants analysed for glutathione. For the pharmacokinetic studies glutathione was determined by HPLC [14]; in the experiments with allyl alcohol/acetaminophen glutathione was determined as total glutathione by an enzymatic cycling assay [15].

Statistics. The results were analysed according to the Mann–Whitney-Wilcoxon test (U-test) or according to Student's *t*-test as indicated. Data are expressed as mean values ± SE; $P \leq 0.05$ was considered to be significant.

RESULTS

In order to determine a suitable time point for the ensuing experiments, the time course of the increase of organ glutathione levels after administration of glutathione *iso*propyl ester was investigated first. Figures 1 and 2 show that i.p.-administration of glutathione *iso*propyl ester (1 g/kg) to starved mice led to a major increase of glutathione in liver and lung which continued for at least 4 hr. Minor glutathione enhancing effects of the administration of the ester were observed in heart after 4 hr, and in brain after 6 hrs post treatment. No significant increase of the GSH-concentration was found in spleen, kidney, muscle, serum and blood cells (data not shown). According to these results, 4 hr were regarded as a suitable time point for further studies, concentrated on those organs where an increase of GSH after administration of the ester was observed.

The dose-dependence of the administration of glutathione *iso*propyl ester on the GSH levels in various organs was studied after a fixed time of administration. Four hours after i.p.-administration of different doses of glutathione *iso*propyl ester (0.2, 0.5, 1 g/kg) a dose-dependent increase of glutathione levels was observed in liver, heart, lung and brain (Table 1). Since the glutathione content of the organs varied somewhat within different animal groups the data are expressed as per cent change compared to the untreated control (100%). At a dose of 1 g/kg of glutathione *iso*propyl ester the highest increase of glutathione was found in liver (166%), lung (164%), heart (121%) after 4 hr (Table 1, Figs 1 and 2), and in brain (133%) after 6 hr (Fig. 2). At doses higher than 1 g/kg no further glutathione increase was observed (data not shown). Spleen, kidney, muscle, serum and blood cell glutathione levels were not significantly altered by the treatment. In no case did the treatment affect the GSH/GSSG ratio, which was found to be about 20. These data show that glutathione *iso*propyl ester is an efficient means to raise organ glutathione levels in mice, and that the integrity of the redox balance is maintained.

In order to investigate the potential pharmacological relevance of this intervention, we examined the effect of glutathione *iso*propyl ester on two widely studied models of hepatotoxicity, i.e. allyl alcohol and acetaminophen. In either intoxication model, liver GSH is severely depleted. It has already been shown that replenishment of liver glutathione by intravenous administration of liposomally entrapped GSH led to full protection against acetaminophen-induced hepatoxicity [16]. In the case of allyl alcohol induced liver injury, a protection by free intravenously given GSH had been found [17].

Table 1. Dose-dependent relative changes of glutathione concentration in several organs of the mouse 4 hr after i.p. administration of glutathione-*iso*propylester (YM737)

Organ	Control = 100%	200 mg/kg % Change	500 mg/kg % Change	1000 mg/kg % Change
Liver (nmol/mg protein)	20.9 ± 0.9 (13)	116 ± 7†	147 ± 10†	166 ± 9†
Heart (nmol/mg protein)	9.4 ± 1.3 (9)	103 ± 11	105 ± 8	121 ± 5†
Lung (nmol/mg protein)	16.3 ± 4.2 (9)	108 ± 6	170 ± 27*	164 ± 9†
Spleen (nmol/mg protein)	25.5 ± 2.9 (6)	97 ± 11	99 ± 9	101 ± 13
Kidney (nmol/mg protein)	11.4 ± 2.3 (6)	92 ± 5	112 ± 8	109 ± 3
Brain (nmol/mg protein)	16.6 ± 0.9 (9)	100 ± 6	98 ± 12	111 ± 22
Muscle (μmol/g organ)	0.9 ± 0.4 (6)	102 ± 9	68 ± 1	74 ± 7
Blood cells (nmol/mg protein)	8.5 ± 1.2 (5)	120 ± 42	135 ± 53	102 ± 3
Serum (μmol/L)	47.2 ± 18.4 (5)	99 ± 8	109 ± 12	59 ± 12†

Data ± SE, * $P < 0.1$; † $P < 0.05$; ‡ $P < 0.01$ (*t*-test).
Number of control animals in parenthesis, otherwise N = 3.

Table 2. Protection by and dose dependence of glutathione *iso*propyl ester (YM737) against allyl alcohol induced liver injury in starved male mice

	GPT [units/L]	GOT [units/L]	SDH [units/L]	GSH + 2-GSSG [nmol/mg protein]	N
Controls	40 ± 3	70 ± 10	120 ± 10	17.0 ± 0.5	4
Allyl alcohol	1740 ± 870	2400 ± 760	1340 ± 360	1.6 ± 0.3	5
Allyl alcohol + YM737 (200 mg/kg)	3840 ± 2290	4140 ± 1640	3110 ± 1340	1.6 ± 0.04	4
Allyl alcohol + YM737 (500 mg/kg)	110 ± 40*	390 ± 210*	30 ± 10*	4.5 ± 1.4*	4
Allyl alcohol + YM737 (1000 mg/kg)	140 ± 70*	330 ± 180*	60 ± 20*	24.6 ± 2.2*	5
Allyl alcohol + YM737 (2000 mg/kg)	80 ± 20*	140 ± 40*	30 ± 10*	38.4 ± 0.8*	5

Dose: allyl alcohol 75 μL/kg i.p., 3 hr after administration of YM737.
Enzyme activities and glutathione were determined 1–4 hr after administration of allyl alcohol.
Data ± SE, * $P < 0.05$ (U-test) compared to disease control, N = number of animals.

Therefore it appeared that allyl alcohol and acetaminophen were suitable models for studying the pharmacodynamics of the ester.

In order to sensitize the animals to allyl alcohol, hepatic glutathione was reduced by keeping the animals on a 10% sucrose diet for 48 hr prior to administration of allyl alcohol. As indicated by serum enzyme release (Table 2) glutathione *iso*propyl ester protected dose-dependently against allyl alcohol induced liver injury. The lowest effective dose was 500 mg/kg. In order to sensitize mice to acetaminophen they were pretreated with benzo[*a*]pyrene for 3 days prior to the experiment as described in Ref. 13. These mice were fully protected by glutathione *iso*propyl ester against acetaminophen-induced liver injury (Table 3). Also here 500 mg/kg was the lowest effective dose. Control experiments with the highest dose of the ester, i.e. 2 g/kg, administered to starved mice, showed no detectable liver injury as assessed by enzyme release (GPT 79 ± 36, GOT 116 ± 59, SDH 54 ± 7 units/L; N = 3).

In both instances a significant correlation between the extent of liver damage and the enhancement of glutathione content following administration of different doses of the ester was obtained. The following correlation coefficients between serum enzyme levels and liver GSH content were found: GPT 0.95, GOT 0.85, SDH 0.99 for acetamino-

Table 3. Protection by and dose dependence of glutathione *iso*propyl ester (YM737) against acetaminophen induced liver injury in starved male mice

	GPT [units/L]	GOT [units/L]	SDH [units/L]	GSH + 2-GSSG [nmol/mg protein]	N
Controls	40 ± 3	70 ± 10	120 ± 10	17.0 ± 0.5	4
Acetaminophen	880 ± 150	880 ± 130	800 ± 200	2.5 ± 0.6	9
Acetaminophen + YM737 (200 mg/kg)	1040 ± 240	1120 ± 260	900 ± 370	1.6 ± 0.8	5
Acetaminophen + YM737 (500 mg/kg)	80 ± 20*	120 ± 40*	100 ± 20*	19.9 ± 4.8*	4
Acetaminophen + YM737 (1000 mg/kg)	70 ± 20*	260 ± 90*	60 ± 20*	31.0 ± 7.0*	8
Acetaminophen + YM737 (2000 mg/kg)	70 ± 6*	140 ± 50*	40 ± 20*	34.5 ± 2.00*	4

Dose: acetaminophen 400 mg/kg i.p., 3 hr after administration of YM737.
Enzyme activities and glutathione were determined 7–10 hr after administration of acetaminophen.
Data ± SE, * $P < 0.05$ (U-test) compared to disease control, N, number of animals.

phen-induced liver injury; GPT 0.84, GOT 0.88; SDH 0.80 in the case of allyl alcohol-induced liver injury. The calculations are based on a multiplicative model ($y = ax^b$) which seemed an appropriate statistical model for the experimental observation, that in both models, usually only extreme responses, i.e. extensive damage or complete protection, were observed (cf. Tables 2 and 3, Refs 13, 16 and 17).

DISCUSSION

The design of this study does not allow distinction between cellular uptake of glutathione *iso*propyl ester, or degradation or export of its metabolite, free glutathione. With this reservation our results clearly demonstrate that within 4–6 hr, intraperitoneal administration of glutathione *iso*propyl ester leads to a net increase of free glutathione in liver, lung, brain and heart in starved mice. These results compare well to those reported earlier for glutathione monomethyl ester [10], glutathione monoethyl ester [11] or glutathione *iso*propyl ester [18]. Some minor differences may be due to different pretreatments such as inhibition of GSH-synthesis with buthionine sulfoximine [10, 11] prior to administration of the ester. The increase of organ glutathione observed here seems to be somewhat slower following the administration of propyl ester compared to ethyl ester [11], with maximum glutathione concentrations reached after 4 hr for the propyl ester and 2 hr for the ethyl ester [11]. In contrast to the ethyl ester the propyl ester was found to increase brain glutathione levels as well. Both differences may be explained in terms of greater hydrophobicity of the propyl ester. Independent of this consideration, it seems that the advantage of the propyl ester consists in the release of a less toxic alcohol after esterase hydrolysis. It should also be noted that glutathione *iso*propyl ester could elevate glutathione only in organs with a high glutathione turnover such as liver and lung which are prone to loss of GSH during starvation [19]. In organs with a lower glutathione turnover which are only marginally depleted of GSH after 24 hr starvation such as spleen and kidney a less pronounced or insignificant effect of glutathione *iso*propyl ester on tissue content of GSH was observed.

The elevation of organ glutathione after administration of glutathione *iso*propyl ester seems to persist for a considerable span of time. Six hours after administration of glutathione *iso*propyl ester there was still a significantly higher glutathione concentration in some organs compared to controls (Figs 1 and 2). Even after the massive challenge with acetaminophen an increase of GSH in the liver persisted for at least 10 hr after administration of the ester (Table 3).

An interesting point resulting from previous studies [16] was the heterogeneity of hepatocytes with respect to their glutathione uptake. When mice had been pretreated with glutathione alone or with liposomally entrapped glutathione prior to acetaminophen, there was an elevation in the overall hepatic glutathione in both instances compared to disease control. But only liposomally entrapped glutathione was able to protect mice against acetaminophen-induced perivenous liver injuy [16]. These experiments suggested that glutathione uptake is not homogeneously distributed within the differently zoned cells in the liver. Allyl alcohol and acetaminophen are characterized by their differential targeting on hepatocyte populations: allyl alcohol acts on periportal [20] whereas acetaminophen acts on perivenous hepatocytes [21]. Therefore it was interesting whether glutathione *iso*propyl ester could protect mice from acetaminophen-induced perivenous liver injury, especially since a protection against acetaminophen-induced lethality had been reported for the methyl ester [10]. Our results demonstrate a protective potential for the glutathione *iso*propyl ester and extend the previous findings in a quantitative manner by showing that it confers full protection against acetaminophen-induced, i.e.

perivenous liver injury (Table 3). The alternative hepatotoxin studied i.e. allyl alcohol causes periportal liver necrosis by cytosolic metabolism resulting in a reactive intermediate. Glutathione *iso*propyl ester was fully protective in this morphologically and biochemically different liver injury model.

From these data we conclude that glutathione *iso*propyl ester when administered intraperitoneally reaches periportal as well as perivenous hepatocytes, where it may counteract adverse effects of hepatotoxic drugs or compounds. In this respect it seems superior to L-2-oxothiazolidine-4-carboxylic acid (ATCA) or glutathione, compounds which were not able to increase glutathione in perivenous hepatocytes, as concluded from their inability to prevent acetaminophen-induced liver injury despite restored GSH content [9]. Compared to liposomally entrapped glutathione, glutathione *iso*propyl ester seems favorable because of a more convenient, i.e. intraperitoneal route of application. In addition, of course, the problem of the fate of liposomal lipid does not exist in this approach of raising GSH levels. Thus, a long lasting effect on organ glutathione, convenient application, the ability to increase GSH in various zones of the liver and its protective effect in different *in vivo* models seem to encourage further studies on the therapeutic potential of glutathione *iso*propyl ester.

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