Biochemical Pharmacology, Vol. 31, No. 22, pp. 3601–3605, 1982.
Printed in Great Britain.

0006-2952/82/223601-05 $03.00/0

DRUG-INDUCED LIPID PEROXIDATION IN MICE—II

PROTECTION AGAINST PARACETAMOL-INDUCED LIVER NECROSIS BY INTRAVENOUS LIPOSOMALLY ENTRAPPED GLUTATHIONE

ALBRECHT WENDEL and HARTMUT JAESCHKE
Physiologisch-chemisches Institut der Universität, Hoppe-Seyler-Str. 1, D-7400 Tübingen, W. Germany
and
MANFRED GLOGER
Boehringer, Mannheim GmbH, Biochemical Research Center, D-8132 Tutzing, W. Germany

(*Received* 5 *March* 1982; *accepted* 11 *May* 1982)

Abstract—If injected intravenously 2 hr before the drug, a dose of more than 175 mg/kg body weight glutathione (0.57 mmol/kg) protected male mice from acute liver necrosis induced by intraperitoneal administration of 400 mg/kg (2.65 mmol/kg) paracetamol. Soluble glutathione yielded a limited, and liposomally entrapped glutathione an optimal dose-dependent protective effect against drug-induced lipid peroxidation (as measured by *in vivo* ethane exhalation) liver necrosis (assessed by serum transaminases) and hepatic glutathione depletion (determined *post mortem*). *N*-Acetylcysteine solution had no effect in this model.

In mammalian cells, the ubiquitous and abundant intracellular thiol glutathione exerts many different metabolic functions including redox-, conjugation- and transpeptidation reactions besides its involvement as an enzymatic cofactor. Its importance for the integrity of the liver has been recognized for a long time [1–4]. In the past, many attempts to ameliorate toxic reactions by administration of glutathione yielded promising results *in vitro* but failed to succeed *in vivo*. The rapid hydrolysis of extracellular glutathione in the circulation has been recognized only recently [5–8]. The finding that the liver is essentially impermeable to glutathione [5] may explain the failure of these attempts.

In large doses, the mild analgesic and antipyretic drug paracetamol (acetaminophen, 4-hydroxy-acetanilid) produces fulminant liver necrosis in animals and man (for a recent review see Ref. [9]). In the previous communication of this series we reported that this type of drug-induced liver necrosis is accompanied in our *in vivo* model by lipid peroxidation and may be the result of it [10]. The extent of liver damage was dependent on the glutathione, selenium, and monooxygenase status [10]. Chemotherapeutic approaches to paracetamol overdosage included administration of glutathione precursors i.e. cysteine or methionine [11] as well as other sulfhydryl nucleophilic compounds. The most successful curative treatments in man seem to have been achieved by the oral [12] or intravenous [13] administration of *N*-acetylcysteine.

A previous communication reports a limited efficacy of i.v. administered glutathione against paracetamol-induced liver necrosis in mice and the lack of efficacy of similar doses of liposomally entrapped glutathione [14]. The actual hepatic glutathione contents after the treatment were unknown. Thus we were prompted to reinvestigate the influence of extracellular glutathione on paracetamol-induced liver necrosis in mice with determination of serum transaminases, *in vivo* lipid peroxidation, and glutathione content in the liver.

MATERIALS AND METHODS

Male albino mice were fed for at least two weeks a low vitamin E diet C 1018 (Altromin, Lage, W. Germany) which had the following composition: 53% starch, 22% casein, 10% sucrose, 3% soybean oil, 6% mineral salts, 2% vitamin mix. On days 4, 3 and 2 before the experiment the animals received a single intraperitoneal injection of 20 mg/kg benzo(*a*)pyrene. 48 hr before the beginning of the experiment they were fed a liquid sucrose diet as described before [10]. Liposomal suspensions and GSH solution were slowly injected into the tail veins of the animals without immobilization or anesthesia. Paracetamol was intraperitoneally injected in dimethylsulfoxide (2 ml/kg) as described [10]. Hydrocarbon exhalation was measured as described [10] over a 4 hr period. The animals were then killed by cervical dislocation and their livers immediately perfused with ice-cold saline. The gall-bladder was removed by freeze-clamping. Glutathione was determined as described [10]. Serum transaminases were determined according to Ref. [15] in heparinized serum obtained from heart-punctured blood. Cytochromes P-450 and b_5 were measured according to Ref. [16]. Glutathione conjugates of paracetamol and free paracetamol were determined by high performance liquid chromatography as described in Ref. [23].

Large unilammellar neutral liposomes (0.1–0.4 μm diameter) containing 40–60 mmol/l. GSH in

5 mmol/l. potassium phosphate buffer (pH = 6.3) and 0.15 mol/l. NaCl were obtained from Boehringer, Mannheim, made according to Ref. [17]: from 66 ml of a freshly prepared chloroform solution containing 20 mg/ml phospholipon-100 (Nattermann, Köln) and 9.61 mg/ml cholesterol, the solvent was evaporated in a 1 l. round-bottom-flask under reduced pressure, 40°, argon atmosphere. The lipid film was redissolved in 150 ml diethylether and 50 ml 0.05 mol/l. phosphate buffer of pH 6.3. 5.0 g reduced glutathione were added. The pH was maintained at pH 6.3 by addition of 5 mol/l. NaOH.

The system was sonicated (Branson Sonifier B 15, output control 9, 30% duty cycle) for 15 min under argon between +1 and +5°. Under reduced pressure the organic solvent was removed at about 18°. During this process the material first formed a gel and then became an aqueous suspension, at a final volume of 50 ml.

Free glutathione was removed from liposomally entrapped GSH by gel filtration on a Sepharose 4 B column (5 × 35 cm). Agglomerated particles were removed by two times filtration on glasswool, and this suspension was concentrated to 10 ml with an Amicon PM 30 membrane. These suspensions contained an average of 25 mg/ml reduced glutathione and 40 mg/ml lipid at a molar phospholipid:cholesterol ratio of about 1:1. Control "empty" liposomes contained buffer, saline and no glutathione.

RESULTS

In order to work with a standardized animal population only mice pretreated with benzo(*a*)pyrene were used. The animals exhibited the following biochemical characteristics compared to untreated controls (in parentheses): 1.26 nmol cytochrome P-450 per mg of total liver protein (0.87); 1.14 nmol cytochrome b_5 per mg protein (0.71); 25 mg microsomal protein per gram liver wet weight (16.7 mg/g).

Taking into account the diurnal variation of hepatic glutathione in rodents, all experiments were begun at 5–9 p.m., when the animals had 25 ± 7 (n = 6) nmol hepatic glutathione per mg protein. The liposome suspensions used contained over 95% of the total GSH as entrapped glutathione with a GSSG content ≤ 15%. They were stable within this margin for at least three months when stored at 4° with exclusion of air at pH = 6.3

Pilot experiments showed that the hepatic GSH levels of mice fed a protein-free diet reached a maximum two hours after the liposomal injection. Therefore, we pretreated mice with different doses of liposomes and challenged the animals two hours later with high doses of paracetamol. Table 1 shows that animals which had not been pretreated exhibited fulminant liver necrosis, lipid peroxidation and hepatic GSH depletion. On the contrary, preinjection with 125 μl or more of liposomal GSH-suspensions (corresponding to a dose of about 10 μmol GSH per animal) greatly decreased the drug-induced liver damage. The experiments demonstrate that the pretreated animals, which originally had a hepatic glutathione content of 25 nmol per mg protein, still had about twice this concentration of hepatic glutathione 4 hr after a challenge of 400 mg/kg paracetamol. Above this dose of liposomally encapsulated GSH, a further increase in hepatic glutathione does not occur. A similar, but inverse, effect is seen on ethane exhalation and serum transaminases. The data indicate a dose-dependent beneficial effect of the GSH-liposomes on drug-induced liver damage.

The dose–response profiles with respect to paracetamol of unpretreated mice were compared with those of animals pretreated with different forms of a fixed dose of GSH. Table 2 contains the results of these experiments with unpretreated animals that received different doses of paracetamol. It shows first of all that at 300 mg/kg paracetamol a drastic decrease of glutathione is accompanied by a large increase in lipid peroxidation. However, a slight serum transaminase elevation is observed at 150 mg/kg, and a considerable one at 225 mg/kg paracetamol even though little ethane is produced at these doses. This means that near the toxicity threshold lipid peroxidation and liver necrosis as estimated by serum transaminases diverge. Secondly, a comparison of ethane exhalation and transaminases of animals not treated with paracetamol compared to animals in Table 1 shows that even at

Table 1. Influence of liposomally entrapped GSH on *in vivo* lipid peroxidation, liver necrosis and *post mortem* hepatic glutathione content in benzo(*a*)pyrene-induced mice treated with 400 mg/kg paracetamol

GSH liposomes[a] (μl)	Ethane exhalation (nmol/kg · 4 hr)	SGPT[c] (U/l.)	Hepatic glutathione[b][c] (nmol/mg)	n	m
0	641 ± 336	3662 ± 2233	6.0 ± 3.0	3	3
50	80 ± 35*	1465 ± 37	7.1 ± 1.0	3	3
125	29 ± 8*	272 ± 88*	50.9 ± 6.3***	3	0
250	34 ± 5*	190 ± 133*	41.5 ± 3.5***	3	0
350	31 ± 5*	279 ± 342*	41.6 ± 4.1***	3	0

n = number of animals.

m = mortality, number of animals that died within 4 hr.

(a) Total volume of a solution containing 25 mg/ml GSH which was intravenously injected 2 hours prior to intraperitoneal injection of paracetamol; (b) expressed in GSH equivalents = GSH + 2 GSSG per mg protein and (c) determined 4 hr after paracetamol administration or after death.

Significances according to Student's t-test: ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05.

Table 2. Dose–response relationship of paracetamol-induced *in vivo* lipid peroxidation, liver necrosis and hepatic glutathione depletion in benzo(*a*)pyrene-induced mice

Dose of paracetamol (mg/kg)	Ethane exhalation (*n*mol/kg · 4 hr)	SGOT (U/l.)	SGPT (U/l.)	Hepatic glutathione (*n*mol/mg)	*n*	*m*
0	13 ± 11	72 ± 11	47 ± 5	28.2 ± 3.4	6	0
75	14 ± 9	70 ± 28	44 ± 5	26.8 ± 2.6	3	0
150	15 ± 11	103 ± 52	89 ± 64	24.7 ± 2.0*	5	0
225	21 ± 13	843 ± 770*	928 ± 795**	20.4 ± 8.3*	8	1
300	569 ± 268***	4967 ± 3374**	5380 ± 3787**	2.0 ± 2.0***	10	3
350	560 ± 175***	6072 ± 2596***	6580 ± 2610***	1.7 ± 2.8***	6	1
400	548 ± 186***	7837 ± 3639***	8576 ± 3887***	0.7 ± 0.5***	8	3
500	356 ± 123***	2735 ± 1126***	3051 ± 1555***	0.9 ± 0.5***	8	2

Table 3. Dose–response relationship of paracetamol-induced *in vivo* lipid peroxidation, liver necrosis and hepatic glutathione depletion in benzo(*a*)pyrene-induced mice which had been treated 2 hr before paracetamol administration by i.v. injection of 200 μl of a solution containing 25 mg/ml GSH

Dose of paracetamol (mg/kg)	Ethane exhalation (*n*mol/kg · 4 hr)	SGOT (U/l.)	SGPT (U/l.)	Hepatic glutathione (*n*mol/mg)	*n*	*m*
0	16 ± 5	68 ± 11	45 ± 7	39.4 ± 10.5	4	0
150	21 ± 7	65 ± 6	52 ± 10	27.5 ± 6.0*	5	0
225	23 ± 6	236 ± 244	308 ± 368	23.6 ± 8.7*	5	0
300	94 ± 66*	1603 ± 2389	1677 ± 2490	10.8 ± 6.3***	5	0
400	278 ± 217*	4265 ± 4481	4562 ± 4620	3.4 ± 1.3***	5	1
500	290 ± 235*	3153 ± 3250	3383 ± 3819	2.1 ± 1.3***	5	1

high doses of GSH-liposomes neither normal transaminase levels nor normal ethane exhalation rates had been reached. At least as far as the ethane production is concerned, this might be caused by the polyunsaturated fatty acids in the liposomal membranes, which had been also taken up into the liver.

The results in Table 3 indicate that preinjection of mice with glutathione solution offers a limited protection of the animals against drug-induced liver necrosis: the drastic increase of lipid peroxidation and transaminases is observed at doses of paracetamol which are about 100 mg/kg higher than in controls (Table 2). The glutathione contents in the animals injected with GSH solution are considerably higher than in the controls, and the paracetamol-induced glutathione depletion becomes manifest at 400 mg/kg paracetamol or more.

The same type of experiment was now done using GSH-filled liposomes for injection. It is evident from Table 4 that this pretreatment rendered the mouse liver much less susceptible to the deleterious effect of drug overdosage. After pretreatment of the animals with these GSH-liposomes, only the highest paracetamol doses produced a GSH depletion. Serum transaminase activities and ethane exhalation were much lower than at the corresponding dose of paracetamol in untreated controls as well as in mice injected with GSH solution. Although the mortality reported is of limited value because the experiment was terminated after 4 hr it is noteworthy that none of the liposome-treated animals died. In a number of animals the total amount of free paracetamol and the glutathione conjugate was determined in the gallbladder. The amount of glutathione conjugate did

Table 4. Dose–response relation of paracetamol-induced *in vivo* lipid peroxidation, liver necrosis and hepatic glutathione depletion in benzo(*a*)pyrene-induced mice which had been treated 2 hr before paracetamol administration by i.v. injection of 200 μl of liposomally entrapped glutathione (25 mg GSH/ml suspension)

Dose of paracetamol (mg/kg)	Ethane exhalation (*n*mol/kg · 4 hr)	SGOT (U/l.)	SGPT (U/l.)	Hepatic glutathione (*n*mol/mg)	*n*	*m*
0	24 ± 7	66 ± 7	43 ± 5	40.0 ± 2.0	5	0
150	30 ± 21	64 ± 11	42 ± 12	36.1 ± 2.6**	6	0
225	28 ± 5	104 ± 67	80 ± 67	34.7 ± 3.2**	6	0
300	25 ± 17	130 ± 76*	106 ± 79	24.8 ± 5.7***	6	0
350	40 ± 13*	132 ± 63*	110 ± 58*	22.7 ± 5.2***	4	0
400	75 ± 45*	285 ± 183*	317 ± 231*	11.7 ± 2.8***	6	0
500	110 ± 56**	182 ± 75**	160 ± 94**	9.0 ± 1.6***	5	0

Table 5. Influence of different types of pretreatments on paracetamol-induced lipid peroxidation, liver necrosis and hepatic GSH depletion of benzo(*a*)pyrene-induced mice

Pretreatment	Ethane exhalation (*n*mol/kg · 4 hr)	SGOT (U/l.)	SGPT (U/l.)	Hepatic glutathione (*n*mol/mg)	*n*	*m*
None	548 ± 186	7837 ± 3639	8576 ± 3887	0.7 ± 0.5	8	3
N-Acetyl cysteine	467 ± 154	7450 ± 2355	8112 ± 2460	0.8 ± 0.2	6	2
GSH-liposomes	75 ± 45***	285 ± 183***	317 ± 231***	11.7 ± 2.8***	6	0
GSH solution[a]	278 ± 217*	4265 ± 4481	4562 ± 4620	3.4 ± 1.3***	5	1
Empty liposomes	700 ± 246	2224 ± 1383*	2946 ± 2130*	1.1 ± 0.8	4	3
GSH solution + empty liposomes	771 ± 135	4808 ± 2192	5659 ± 2931	1.0 ± 0.7	4	2

Pretreatment by i.v. injection of the same volume 2 hr prior to i.p. injection of 400 mg/kg paracetamol (250 μl per animal; none: isotonic saline; GSH solution: 25 mg GSH/ml; *N*-acetylcysteine: 13.2 mg/ml).

(a) One animal in this series did not respond at all to the paracetamol intoxication.

not depend on the dose of paracetamol above 150 mg/kg and was independent of the pretreatment (206 ± 102 nmol conjugate per bladder, 17 ± 9 μl biles, $n = 39$). The gall-bladders of the unpretreated mice contained 92.5 ± 4.4% ($n = 13$) GSH conjugate, those of the animals which had received GSH solution 91.6 ± 5.9% ($n = 16$) and those of the mice injected with GSH-liposomes 92.6 ± 5.7% ($n = 10$). This indicates that the pretreatment had no influence on phase II metabolism of paracetamol.

A final experiment was performed to learn whether packaging GSH-liposomes has any effect. The results (Table 5) indicate that pretreatment of the animals with empty liposomes had no significant effect on ethane exhalation and GSH depletion; however, it decreased or delayed the transaminase release from the liver to some extent. On the other hand, the administration of empty liposomes simultaneously with soluble glutathione seemed to diminish the effect of the glutathione treatment on ethane exhalation and glutathione depletion, but did not significantly reduce the transaminase levels. More important the data indicate that empty liposomes alone do not interfere with ethane exhalation and the extent of paracetamol-induced glutathione depletion. However, a significant decrease of serum transaminases by pretreatment with empty liposomes alone is observed at this fixed treatment regimen. Finally the data in Table 5 show that an analogous treatment of the animals with *N*-acetylcysteine was ineffective in our system.

DISCUSSION

Previous work showed that neutral liposomes containing equimolar amounts of phospholipid and cholesterol should be suitable to prevent leakage of a relatively small molecule like GSH *in vitro* [18] as well as to minimize lipid transfer to high density lipoproteins [19]. They are non-toxic with an LD_{50} in the range of 10 g/kg [20], attain a half-life of ~2 hr in rodents and are taken up in mice by liver, spleen and kidney [21]. The results obtained in this work are fairly consistent with these observations. However, our present knowledge on the interaction of liposomes with the highly differentiated cells of mammalian organs seems too limited to allow further conclusions about the site of uptake or distribution. Thus this discussion remains confined to the observed pharmacological effects and their biochemical correlates. The pharmacokinetics and organ distribution of various forms of intravenously administered glutathione will be reported in a separate communication [22]. The results reported here clearly demonstrate that intravenous injection of glutathione solution into mice results in an increase in hepatic glutathione due to *de novo* synthesis [5] yielding limited prevention of paracetamol-induced liver necrosis. Pretreatment with liposomally entrapped glutathione leads to a similar glutathione increase in the liver but yields much better protection against this type of drug-induced liver necrosis. At the first glance, the effect of both treatments seem to be due to an enhanced hepatic glutathione content. This statement needs a more detailed discussion: mouse liver contains a total of about 6 μmol glutathione under the dietary conditions used here. Injection of 16 μmol glutathione led to a hepatic glutathione content from initially 6 up to 8.6 μmol glutathione, irrespective of whether it was injected in free or encapsulated form (Tables 2–4 first line). The biological potency of this liver glutathione, however, was extremely different, i.e. it is highly unlikely that an even distribution within the organ took place. This different availability is also reflected in the dose-dependence of the GSH depletion: if we consider the data for a dose of 300 mg/kg paracetamol, comparison of Tables 2–4 shows a depletion to 2 nmol per mg glutathione in untreated mice but only to 11 nmol/mg in GSH-treated and to 25 nmol/mg in GSH-liposomes-treated animals. The observation that no significantly enhanced conjugate formation is found in any of the differently pretreated animals rules out a feasible apparent protection by a block in drug metabolism caused by the pretreatment. On the other hand this finding includes the possibility that a GSH-dependent reaction other than phase II-conjugation of the drug to form a premercapturic acid may be responsible for the protection, i.e. reduction of hydroperoxide *via* glutathione peroxidase [10].

Our observations are in agreement with previous work [14] as far as the limited protection by free glutathione is concerned. They differ from the find-

ings reported in Ref. [14] with respect to the effect of liposomes, which were there found to offer even less protection than the faint amount observed for free glutathione. This divergence might be due to several reasons:

(1) The liposomes used in the earlier work were different. They were small, probably multilammelar vesicles and did not contain cholesterol to prevent leakage of the entrapped compound.

(2) A dose of 4×112 to 124 mg/kg was given 15–105 min after administration of 500 mg/kg paracetamol, i.e. a curative effect was investigated. Here we used a single dose of about 175 mg/kg GSH and looked at a preventive effect.

(3) In the earlier studies GSH was entrapped at pH 7.6 where autoxidation to GSSG is possible.

Further investigations are needed with respect to different treatment regimens of liposomally entrapped GSH. The ultimate aim, human treatment after overdosage with suicidal intention, requires a curative treatment after admission, which is usually at least several hours after drug intake.

Acknowledgements—Helpful suggestions made by Dr. R. F. Burk, San Antonio, Texas, and Dr. M. Nelböck, Tutzing, as well as technical assistance by M. Fausel and W. Hillesheim, Tübingen, and J. Heinle, Tutzing, are gratefully acknowledged.

REFERENCES

1. *Glutathione: Metabolism and Function* (Eds. I. M. Arias and W. B. Jakoby). Raven Press, New York (1976).
2. *Functions of Glutathione in Liver and Kidney* (Eds. H. Sies and A. Wendel). Springer, New York (1978).
3. D. Reed and P. Beatty, *Rev. Biochem. Toxicol.* **2**, 213 (1980).
4. N. S. Kosower and E. M. Kosower, in *Free Radicals in Biology* (Ed. W. A. Pryor), Vol. II, p. 55. Academic Press, New York (1976).
5. R. Hahn, A. Wendel and L. Flohe, *Biochim. biophys. Acta* **639**, 324 (1978).
6. D. Häberle, A. Wahlländer and H. Sies, *FEBS Lett.* **108**, 335 (1979).
7. K. Ormstad, T. Lästbom and S. Orrenius, *FEBS Lett.* **112**, 209 (1980).
8. A. Wendel and P. Cikryt, *FEBS Lett.* **120**, 209 (1980).
9. M. Black, *Gastroenterology* **78**, 382 (1980).
10. A. Wendel and S. Feuerstein, *Biochem. Pharmac.* **30**, 2513 (1981).
11. J. R. Mitchell, S. S. Thorgeirsson and W. Z. Potter, *Clin. Pharmac. Ther.* **16**, 676 (1974).
12. R. G. Peterson and B. H. Rumack, *J. Am. Med. Ass.* **237**, 2406 (1977).
13. L. F. Prescott, J. Park and A. Ballantine, *Lancet* **ii**, 432 (1977).
14. A. Malnoe, A. Louis, M. S. Benedetti, M. Schneider, R. L. Smith, L. Kreber and R. Lam, *Biochem. Soc. Trans.* **3**, 730 (1975).
15. H. U. Bergmeyer, *Verlag Chemie*, Vol. I. Weinheim, Germany (1970).
16. R. W. Estabrook and J. Werringloer, in *Methods in Enzymology* **52**, 212 (1978).
17. T. Szoka and D. Papahadjopoulos, *Proc. natn. Acad. Sci.* U.S.A. **75**, 4194 (1978).
18. C. Kirby, J. Clarke and G. Gregoriadis, *FEBS Lett.* **111**, 324 (1980).
19. G. Gregoriadis, *Nature, Lond.* **283**, 814 (1980).
20. R. L. Juliano, *Trends Pharmac. Sci.* **8**, 39 (1981).
21. G. Gregoriadis and J. Senior, *FEBS Lett.* **119**, 43 (1980).
22. A. Wendel and H. Jaeschke, *Biochem. Pharmac.* **31**, 3607 (1982).
23. A. R. Buckpitt, D. E. Rollins and J. R. Mitchell, *Biochem. Pharmac.* **28**, 2941 (1979).