Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor

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Glutathione (GSH) protects rat liver microsomes against ascorbic acid (0.2 mM)/ferrous iron (10 μM)-induced lipid peroxidation for some time. The inhibitory effect of GSH is concentration-dependent (0.1–1.0 mM). Our data suggest that GSH acts by preventing initial radical formation rather than via radical scavenging or GSH–peroxidase activity. A labile GSH-dependent factor is involved in the inhibition of microsomal lipid peroxidation by GSH, inasmuch as heating the microsomes abolishes the GSH effect. We found that besides heating, lipid peroxidation also destroys the GSH-dependent factor. Consequently, continuous radical stress will produce lipid peroxidation, despite the presence of GSH. Moreover, a detrimental effect of in vivo-induced lipid peroxidation (CCl_4-treatment) on the GSH-dependent factor was observed. The implications of the present data for the genesis of and the protection against peroxidative damage are discussed.

Lipid peroxidation *Glutathione* *Vitamin C* *Ferrous iron* *Liver microsome*

1. INTRODUCTION

Lipid peroxidation has been implicated as a major process in cellular damage [1]. Reduced glutathione (GSH) is considered to be a potent inhibitor of lipid peroxidation, but the mechanisms by which it carries out this function are not clear. The protective action of cytosolic glutathione peroxidase and glutathione transferase has been questioned. Both enzymes do not reduce microsomal lipid peroxides at a measurable rate [2,3]. It has also been stated that the glutathione-dependent cytosolic factor which inhibits lipid peroxidation in biological membranes does so by preventing radical attack on the polyunsaturated fatty acids. Here, the activity of GSH in microsomal lipid peroxidation is evaluated.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Glutathione and thiobarbituric acid were obtained from Merck (Darmstadt). All other chemicals used were of analytical grade purity.

2.2. *Preparation of microsomes*

Male Wistar rats (T.N.O., Zeist), 200–250 g, were killed by decapitation. Livers were removed and homogenized (1:2, w/v) in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10000 × g (20 min at 4°C). Subsequently the supernatant was centrifuged at 10000 × g (20 min) and again at 65000 × g (60 min). The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml) and stored at −80°C. Before use the microsomes were thawed and diluted 5-fold with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and washed twice with cen-

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trifugation at 115000 × *g* (40 min). Finally the pellet was resuspended in the Tris buffer and used.

2.3. *Pretreatment of rats*

Carbon tetrachloride (CCl_4) pretreatment consisted of the oral administration of a CCl_4/corn oil (50%, v/v) solution (5 ml/kg). Water and food were provided ad libitum. After 18 h liver microsomes were prepared as in section 2.2, but were less diluted in order to get the same protein concentration as in the control incubations.

Starvation consisted of a period of 18 h food deprivation before decapitation. Water was given ad libitum. Phorone treatment was as in [4], 250 mg/kg i.p., and decapitation after 2 h.

2.4. *Incubation conditions*

Microsomes (final conc. 1/8 g liver/ml) were incubated at 37°C, with shaking air being freely admitted in Tris–HCl/KCl (50 mM/150 mM, pH 7.4). Ascorbic acid and GSH were neutralized with KOH before addition. Reactions were started by adding a freshly prepared $FeSO_4$ solution.

2.5. *Spectral measurements*

Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA) reactive material. An aliquot of the incubation (0.3 ml) was stopped by mixing with ice-cold TBA–trichloroacetic acid–HCl–butylhydroxytoluene (BHT) solution (2 ml). After heating (15 min, 80°C) and centrifugation (15 min) the absorbance at 535 nm *vs* 600 nm was determined. The TBA–trichloroacetic acid–HCl solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8% w/v in 0.125 N HCl). To 10 ml TBA–trichloroacetic acid–HCl 1 ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

Thiol determination was performed after [5]. Corrections were made for the absorbance produced by iron. Microsomal protein was assayed as in [6], using bovine serum albumin as standard.

3. RESULTS

GSH protects rat liver microsomes against lipid peroxidation promoted by ascorbic acid and ferrous iron in a concentration-dependent manner

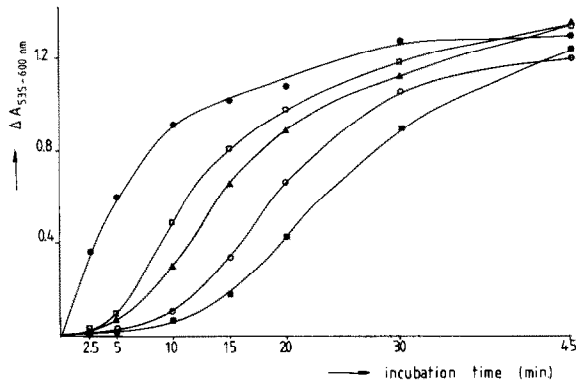


Fig.1. Influence of GSH on the time course of lipid peroxidation. Microsomes (1.3 mg protein/ml) were incubated with 0.2 mM ascorbate. [GSH] was 0 mM (●), 0.1 mM (□), 0.2 mM (▲), 0.5 mM (○), 1 mM (■). Reactions were started with addition of Fe^{2+} (10 μM). Data represent one example out of at least 4 duplicate expt.

(fig.1). This protective effect of GSH is abolished by heating the microsomes in boiling water for 90 s (fig.2). This indicates the involvement of (a) microsomal heat-labile factor(s) in the GSH effect [7].

To investigate the GSH consumption during

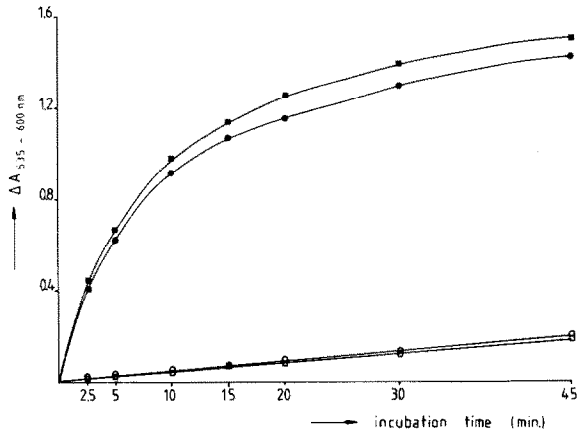


Fig.2. Time course of lipid peroxidation. The reaction mixture consisted of heated microsomes (1.3 mg protein/ml, before heating) 0.2 mM ascorbate (●), 0.2 mM ascorbate and 1 mM GSH (■), no addition (○), 1 mM GSH (□). All reactions were initiated with addition of Fe^{2+}. Data represent one example out of at least 4 duplicate expt.

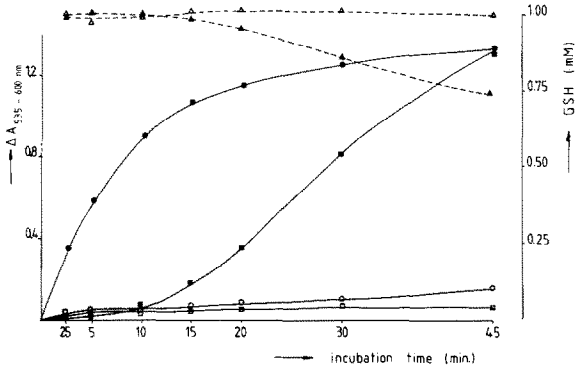


Fig.3. Time course of lipid peroxidation (——) and thiol concentrations (···). Microsomes (1.2 mg protein/ml) were incubated with 0.2 mM ascorbate (●), 0.2 mM ascorbate and 1 mM GSH (■) with (▲) as the corresponding thiol concentration, no addition (○), 1 mM GSH (□), with (△) as the corresponding thiol concentration. All reactions were started with addition of Fe^{2+}. Data represent one example out of 2 duplicate experiments.

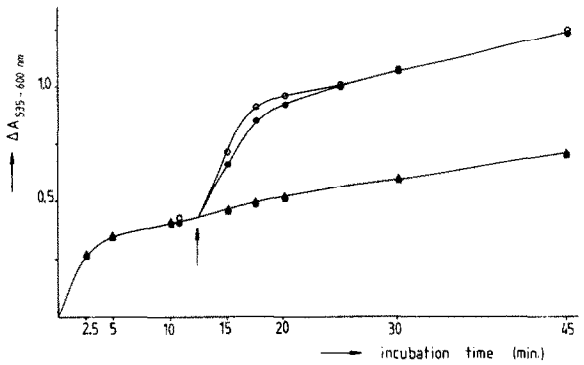


Fig.4. Time course of lipid peroxidation. Microsomes (1.1 mg protein/ml) were incubated with 0.05 mM ascorbate. The reaction was started with the addition of Fe^{2+} (10 μM). After 12.5 min the following additions were made: 0.05 mM ascorbate and 1 mM GSH (●), 0.05 mM ascorbate and the same volume of Tris buffer which was needed for GSH (○), 1 mM GSH (■), none (△). Appropriate corrections were made for changes in volume.

lipid peroxidation the TBA-test has been combined with a thiol determination (fig.3). The results of fig.1 and 3 show that GSH delays but does not prevent lipid peroxidation. After a lag time a strong increase in TBA-reactive material is observed, whereas GSH-levels remain relatively high. In order to explain the rise in lipid peroxidation which eventually occurs, notwithstanding the presence of GSH, we thought that the GSH-dependent lipid peroxidation inhibiting factor(s) may be destroyed during initial but persistent stress. To investigate the notion, the GSH protection after moderate lipid peroxidation has been examined (fig.4). No protection by GSH is observed (fig.4) indicating that the factor(s) is(are) indeed destroyed during lipid peroxidation.

Furthermore, we investigated the stability of the factor(s) in vivo. Pretreatment of rats with CCl_4 resulted in a diminished protective effect of GSH in microsomal incubates compared with the effect observed in hepatic microsomes obtained from oil pretreated (control) rats. Also, GSH depletion induced by pretreatment with phorone [4] or by starvation [8], reduced the protection by GSH in microsomes, compared with results established in hepatic microsomes from untreated rats (not shown).

4. DISCUSSION

The effect of GSH on ascorbate/iron induced microsomal lipid peroxidation is studied. Ascorbic acid (0.2 mM) does not stimulate lipid peroxidation in the absence of iron, nor does ferric iron (10 μM) in the absence of ascorbic acid. Ascorbic acid added in combination with ferric iron results in substantial lipid peroxidation (not shown). Taking this into account, as well as the fact that ferrous iron is capable of promoting lipid peroxidation (fig.2,3), the role of ascorbic acid appears to be maintenance of iron in the reduced (ferrous) form [9]. The stimulation of lipid peroxidation produced by GSH in a system containing heated microsomes, ascorbic acid and ferrous iron (fig.2) can be explained by the regeneration of ascorbic acid by GSH, inasmuch as GSH is able to reduce dehydroascorbic acid to ascorbic acid. This reaction however, has probably no significance in vivo [10]. Without ascorbic acid, GSH does not stimulate, indicating that GSH itself does not reduce trivalent iron at a measurable rate [11] (fig.2). In the system containing ascorbic acid, ferrous iron and microsomes, ascorbic acid becomes irreversibly oxidized since it is shown that addition of GSH, 12.5 min after ferrous iron, does not

enhance formation of TBA-reactive material (fig.4) [12].

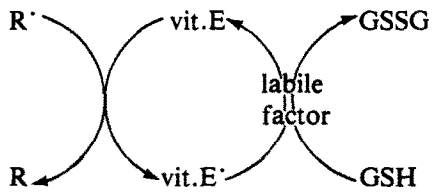
Microsomal lipid peroxidation is inhibited by GSH (fig.1). GSH acts by preventing radical formation. The protection by GSH is not due to radical scavenging or GSH-peroxidase activity because in that case:

(i) A decline in GSH levels should be observed during the lag phase. The decrease in GSH levels per se will lead to enhancement of lipid peroxidation. However, we did not observe a substantial consumption of GSH (fig.3) which would bring GSH levels under scavenging concentration.

(ii) Maximal lipid peroxidation should not be the same in peroxidising microsomes with and without GSH. Our results however demonstrated the same extent of peroxidation in both systems (fig.1,3).

Protection by GSH proceeds via a heat-labile microsomal factor (fig.2). Apparently, also a continuous radical stress (fig.1,3) or lipid peroxidation (fig.4) affects the GSH-dependent factor.

Recently the influence of α-tocopherol (vitamin E) on ascorbate/iron-induced peroxidation in liposomes was thoroughly studied [13]. One molecule of α-tocopherol was found to be able to protect even 220 molecules of polyunsaturated fatty acids. Moreover, it was stated that α-tocopherol may prevent radical formation without undergoing any measurable oxidation itself. Our results indicate that GSH also acts via inhibition of radical formation. It is therefore tempting to suggest an interaction between the water-soluble GSH and the lipid-soluble vitamin E comparable to the presumed linkage between vitamins C and E [14]:



Liver microsomes of vitamin E-deficient rats are devoid of a protective effect by GSH [15]. The conclusion was therefore reached that inhibition by GSH is vitamin E-dependent. However, vitamin E depletion has been found to produce an enhanced ethane exhalation in rats [16]. Since the latter is an index of lipid peroxidation, the inability of GSH to protect might be explained by the destruction of the labile factor. In our opinion so far no direct evidence for the vitamin E dependency of the protective effect by GSH has been adduced. If vitamin E does play a role, it is certainly not the only component since in heated microsomes the inhibition by GSH is eliminated, whereas heating has no effect on the vitamin E level [15].

Attempts have been made to isolate GSH-dependent protective factor(s). In [17,18] the investigators claimed to have isolated a GSH-dependent peroxidation inhibiting protein (PIP). Peculiarly however, they observed no protective effect by GSH in intact microsomes. Furthermore the action of PIP was ascribed to its capacity to reduce hydroperoxide derivatives of phospholipids, at the expense of GSH. Our data suggest another protective mechanism by GSH.

We also demonstrated the instability of the GSH-dependent protective factor(s) in vivo. A decreased protection by GSH is obtained in vitro, using hepatic microsomes from CCl_4-pretreated rats (fig.5).

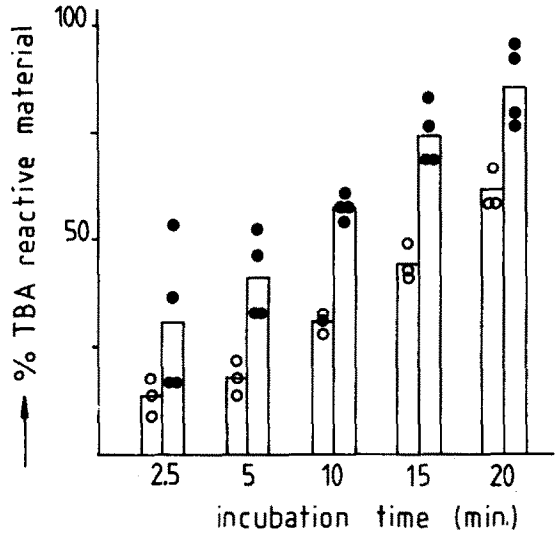


Fig.5. Percentage change in lipid peroxidation using a system containing microsomes, 0.2 mM ascorbate, 1 mM GSH and 10 μM Fe^{2+} compared with the lipid peroxidation without GSH, measured at several incubation times. Each data point represents the mean of a duplicate experiment in which liver microsomes were prepared from one rat. Oil-pretreated control rats (○) (1.3–1.6 mg protein/ml) and CCl_4-pretreated rats (●) (1.2–1.9 mg protein/ml).

The chain reaction of microsomal lipid peroxidation might be initiated after destruction of the GSH-dependent peroxidation inhibiting factor(s). Our results suggest that in that stage the progressive adverse membrane damage cannot be reversed via a direct or indirect increase of GSH levels. This is of importance in order to be able to provide adequate measures against hepatotoxic effects of many xenobiotics which give lipid peroxidation.

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