Inhibition of microsomal lipid peroxidation by glutathione and glutathione transferases B and AA

Role of endogenous phospholipase A₂

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(Received 9 November 1983/Accepted 10 February 1984)

Lipid peroxidation *in vitro* in rat liver microsomes (microsomal fractions) initiated by ADP-Fe³⁺ and NADPH was inhibited by the rat liver soluble supernatant fraction. When this fraction was subjected to frontal-elution chromatography, most, if not all, of its inhibitory activity could be accounted for by the combined effects of two fractions, one containing Se-dependent glutathione (GSH) peroxidase activity and the other the GSH transferases. In the latter fraction, GSH transferases B and AA, but not GSH transferases A and C, possessed inhibitory activity. GSH transferase B replaced the soluble supernatant fraction as an effective inhibitor of lipid peroxidation *in vitro*. If the microsomes were pretreated with the phospholipase A₂ inhibitor *p*-bromophenacyl bromide, neither the soluble supernatant fraction nor GSH transferase B inhibited lipid peroxidation *in vitro*. Similarly, if all microsomal enzymes were heat-inactivated and lipid peroxidation was initiated with FeCl₃/sodium ascorbate neither the soluble supernatant fraction nor GSH transferase B caused inhibition, but in both cases inhibition could be restored by the addition of porcine pancreatic phospholipase A₂ to the incubation. It is concluded that the inhibition of microsomal lipid peroxidation *in vitro* requires the consecutive action of phospholipase A₂, which releases fatty acyl hydroperoxides from peroxidized phospholipids, and GSH peroxidases, which reduce them. The GSH peroxidases involved are the Se-dependent GSH peroxidase and the Se-independent GSH peroxidases GSH transferases B and AA.

The hepatic metabolism of certain drugs, carcinogens and endogenous lipids may result in the release of reactive free radicals capable of initiating lipid peroxidation in cellular membranes (Mitton *et al.*, 1971; Plaa & Witschi, 1976; Slater, 1979; Floyd, 1980; Müller *et al.*, 1981; Tappel & Dillard, 1981; Mitchell *et al.*, 1982). If it were to proceed uninhibited, lipid peroxidation of cellular membranes would cause extensive cellular damage, and a number of factors have been implicated in providing protection, including enzymes with GSH peroxidase activity and the free-radical trap, vitamin E. The importance of the former is demonstrated by the effect of GSH depletion, which leads to increased lipid peroxidation *in vivo* during drug metabolism (Wendel *et al.*, 1979;

Anundi *et al.*, 1979; Ekstrom & Hogberg, 1980; Younes & Siegers, 1981; Wendel & Feuerstein, 1981; Levine, 1982).

Most studies of the mechanism of protection by GSH peroxidases have been studies *in vitro* in which lipid peroxidation in microsomal membranes has been initiated with reduced iron chelates (Christophersen, 1966, 1968; Wills, 1969*a,b*; McCay *et al.*, 1976; Gibson *et al.*, 1980; Burk *et al.*, 1980; Wright *et al.*, 1981). Using such a system, Burk *et al.* (1980) showed that partially purified preparations of GSH transferases from rat liver, which also exhibited GSH peroxidase activity (Prohaska & Ganther, 1977), were significant inhibitors of lipid peroxidation. However, Gibson *et al.* (1980), studying lipid peroxidation induced in heat-treated membranes, failed to obtain inhibition by the GSH transferase fraction, and pointed out (see also van Deenen, 1965; Sevanian

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

et al., 1981; Flohé, 1982) that the GSH peroxidases of the soluble supernatant could only react with free fatty acyl hydroperoxide which had been released from peroxidized phospholipid by phospholipase action.

In the present work we have further analysed the inhibition of lipid peroxidation by GSH alone and GSH-dependent factors present in the soluble supernatant of rat liver, including purified GSH transferase B. Part of this work has been published in a preliminary report (Tan *et al.*, 1983).

Experimental

Materials

The following compounds were obtained from Sigma, Poole, Dorset, U.K.: ADP (grade III), bovine serum albumin, butylated hydroxytoluene (2,6-di-*t*-butyl-*p*-cresol), glucose 6-phosphate, GSH, horse cytochrome *c*, porcine pancreatic phospholipase A₂, bovine liver phosphatidylethanolamine, lysophosphatidylethanolamine and 2-thiobarbituric acid (4,6-dihydroxy-2-thiopyrimidine). 2',7'-Dichlorofluorescein and *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide, a precursor to diazomethane, were from Aldrich, Gillingham, Dorset, U.K. Glucose-6-phosphate dehydrogenase, GSH reductase and NADPH were obtained from Boehringer, Lewes, East Sussex, U.K. FeCl₃ was from Fisons, Loughborough, Leics., U.K. 1-Chloro-2,4-dinitrobenzene and 1,1,3,3-tetramethoxypropane were from Fluorochem, Glossop, Derbyshire, U.K. H₂O₂ (30%, w/v) was from BDH, Poole, Dorset, U.K. Sephadex G-25 and G-100, and Sephacryl S-200, were from Pharmacia, London W.5, U.K. Hydroxyapatite HTP was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Phenobarbitone sodium was from Evans Medical, Liverpool, U.K., and *p*-bromophenacyl bromide was from Pierce and Warriner, Chester, Cheshire, U.K. Phosphatidylserine and egg lecithin were from Lipid Products, South Nutfield, Redhill, Surrey, U.K. All other reagents used were of analytical grade.

Methods

Buffers used. Two buffers designated A and B were used in the present study. Buffer A was 50 mM-Tris adjusted to pH 7.5 with HCl. Buffer B contained physiological concentrations of K⁺, Na⁺, Mg²⁺, Cl⁻ and PO₄³⁻ (Eveleth, 1937; Fenn, 1939; Rapoport, 1945; Bernstein, 1954) and had the composition 6 mM-KH₂PO₄/10 mM-NaCl/90 mM-KCl/3 mM-MgCl₂ and was adjusted to pH 7.5 with KOH.

Preparation of microsomes (microsomal fractions) and soluble supernatant fractions. Male Wistar rats,

inbred in the Courtauld Institute of Biochemistry and weighing 200–250 g, were fed on a standard diet (Diet 86; E. Dixon and Sons, Ware, Herts., U.K.) and for 5–7 days before being killed were given drinking water containing 0.1% sodium phenobarbitone. After the rats had been killed by cervical dislocation, microsomes and soluble supernatant fractions were prepared in 0.25 M-sucrose as described by Burk *et al.* (1980). The soluble supernatant was exhaustively dialysed against either Buffer A or Buffer B in order to decrease its GSH content to minimal levels. Determination of GSH levels in dialysed soluble supernatant fraction by the method of Habeeb (1972) gave levels of the order of 1 μM.

Induction of peroxidation. (a) Untreated microsomes. Microsomes from 0.4 g of rat liver were added to one of the following two incubation mixtures. The first was based on that described by Svingen *et al.* (1979) and had the composition: 0.1 mM-FeCl₃/1.1 mM-ADP/0.3 mM-NADPH in Buffer A. The second, where concentrations of ADP and NADPH were based on values for their concentrations in rat liver (Rapoport, 1945; Glock & McLean, 1955), had the composition 0.1 mM-FeCl₃/3 mM-ADP/0.3 mM-NADPH in Buffer B. In both cases ADP and FeCl₃ were first dissolved together in distilled water before addition to the rest of the incubation mixture in order to ensure that the ADP-Fe³⁺ chelate was formed rather than mixed iron hydroxides. Unless specified otherwise, incubations were carried out at 37°C in air for 5 min. When time-course studies over a longer period of time were made, NADPH was regenerated with glucose 6-phosphate and glucose-6-phosphate dehydrogenase as described by Burk *et al.* (1980).

(b) Heat-treated microsomes. Peroxidation of heat-treated microsomes was initiated by a modification of the method of Gibson *et al.* (1980). Microsomes were suspended in Buffer A and heated at 100°C for 10 min. A sample of heat-treated microsomes equivalent to 0.4 g of liver was added to an incubation mixture with the composition 4 mM-ADP/12 μM-FeCl₃/0.6 mM-sodium ascorbate in Buffer A. Incubations were carried out in air at 37°C for 5 min.

(c) Microsomes treated with *p*-bromophenacyl bromide. Microsomes equivalent to 8 g of liver, suspended in either Buffer A or B, were allowed to react with *p*-bromophenacyl bromide at a concentration of 1 mM for 15 min at 20°C. Unchanged *p*-bromophenacyl bromide was removed by reaction with 5 mM-GSH for 120 min, which formed a GSH adduct. The GSH conjugate, excess GSH and its oxidation product, GSSG, were removed by dialysis. Control microsomes were incubated and dialysed as above, except that *p*-bromophenacyl

bromide was absent. Peroxidation of these microsomes was brought about as described above for untreated microsomes.

Measurement of lipid peroxidation *in vitro*. Malonaldehyde determination. Malonaldehyde production was the most commonly used indicator of lipid peroxidation and measured by a modification of the method of Ernster & Nordenbrand (1967). The incubation mixture (0.5 ml) was mixed with 2 ml of reagent containing 10% (w/v) trichloroacetic acid, 0.25% (w/v) thiobarbituric acid and 0.1% (w/v) butylated hydroxytoluene and heated for 15 min at 80°C. To avoid interference from turbidity, the coloured complex was extracted into 2.5 ml of butanol, centrifuged, and the A_{532} of the butanol phase measured.

Synthetic malonaldehyde was generated by acid hydrolysis of 1,1,3,3-tetramethoxypropane. Neither the soluble supernatant nor GSH transferases at concentrations used in experiments designed to inhibit lipid peroxidation affected its assay. This showed that protein with the potential of forming Schiff bases did not interfere with the determination of malonaldehyde.

Phospholipid analysis. Phosphatidylethanolamine is rich in unsaturated fatty acyl side chains and therefore likely to be affected by lipid peroxidation. Both total phosphatidylethanolamine and its fatty acyl composition were used to monitor lipid peroxidation.

Microsomal phospholipid was extracted by the method of Folch *et al.* (1957) and separated by t.l.c. as described by Skipski *et al.* (1962). The phospholipids were detected with dichlorofluorescein and identified by comparison with standards. Silica containing the phosphatidylethanolamine spot was scraped off the chromatogram and washed twice with 5 ml of 7% (w/v) NH_3 to remove dichlorofluorescein. Total phosphatidylethanolamine was quantified by analysis of organic phosphate by the method of Bartlett (1959). The fatty acid composition of phosphatidylethanolamine was determined by saponification, methylation and analysis of the resulting methyl esters on g.l.c. by the method of Marai & Kuksis (1973).

Measurement of enzyme activities. GSH transferase activities were assayed with 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene or *trans*-4-phenylbut-3-en-2-one as substrates by the method of Habig *et al.* (1974). GSH peroxidase was measured with H_2O_2 as substrate by the method of Paglia & Valentine (1967) and cumene hydroperoxide by the method of Prohaska & Ganther (1976). Phospholipase A_2 activity was determined in the presence of 1 mM- CaCl_2 by measuring the loss of phosphatidylethanolamine in microsomal lipid extracts as described above.

Fractionation of rat liver soluble supernatant

fraction by chromatography. (a) Zonal elution. A 5 ml portion of a soluble supernatant fraction obtained by centrifuging a 1:1 (w/v) rat liver homogenate at 105 000 g_{av} for 1 h was applied to a Sephadex G-100 column (2.5 cm $\times$ 90 cm) and eluted with either Buffer A or Buffer B. Fractions of volume 4 ml were collected.

(b) Frontal elution. A portion (50–170 ml) of soluble supernatant fraction obtained by centrifuging a 1:3 (w/v) rat liver homogenate at 105 000 g_{av} for 1 h was applied to a Sephacryl S-200 column (1.5 cm $\times$ 85 cm) containing Buffer B made 5 mM with respect to GSH. When all the soluble supernatant fraction had entered the column, Buffer B + GSH was applied until elution of all components of the soluble supernatant fraction was complete; 1.5 ml fractions were collected.

In this technique (Novák & Janák, 1975) the volume of soluble supernatant applied is larger than the included volume of the column. Each component of the soluble supernatant fraction emerges from the column as a front and reaches its concentration in the original soluble supernatant fraction at its elution volume. Thereafter it continues to be eluted at this concentration. When all the components of the soluble supernatant have emerged, the eluate has the composition of the soluble supernatant originally applied to the column. A second series of profiles is obtained as successive components cease to be eluted in the order in which their fronts first appeared.

Purification of GSH transferases. Purified hepatic GSH transferases were prepared from phenobarbital-induced rats as described by Beale *et al.* (1983).

Inhibition of lipid peroxidation. The following materials in a range of concentrations were tested for their ability to inhibit lipid peroxidation in Buffers A and B: GSH, exhaustively dialysed whole soluble supernatant, fractions from zonal-elution and frontal-elution chromatography, and purified GSH transferases.

Results

GSH-dependent inhibition of lipid peroxidation by the soluble supernatant fraction

Table 1 shows that, in the presence, but not in the absence, of GSH, the dialysed soluble supernatant fraction causes inhibition of malonaldehyde formation by incubation of microsomes undergoing ADP- Fe^{3+} -plus-NADPH-initiated lipid peroxidation. This effect is concentration-dependent.

At concentrations of soluble supernatant fraction approximately half of that in the cell, the inhibition of lipid peroxidation in this '*in vitro*' system is 90%.

Table 1. *Effect of the soluble supernatant fraction and GSH transferase B on lipid peroxidation induced in microsomes by ADP-Fe³⁺ plus NADPH*

The incubation mixture contained microsomes (5 mg protein/ml), 3 mM-ADP, 0.1 mM-FeCl₃ and 0.1 mM-NADPH in buffer B. Incubation was at 37°C for 5 min. Where indicated, soluble supernatant fraction (11–28 mg of protein/ml), GSH transferase B (0.8–5 mg/ml) and 2.5 mM-GSH were added to the assay mixture. Peroxidation was estimated (mean ± s.d.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with four other microsomal preparations gave similar results.

Additions	Peroxidation	
	(nmol of malonaldehyde/min per ml)	(% of control)
None	4.10 ± 0.33	100
GSH (2.5 mM)	1.96 ± 0.22	49
Soluble supernatant fraction		
28 mg of protein/ml	4.18 ± 0.06	102
28 mg of protein/ml, + GSH	0.53 ± 0.12	13
22 mg of protein/ml, + GSH	1.15 ± 0.11	28
11 mg of protein/ml, + GSH	1.52 ± 0.06	37
GSH transferase B		
5 mg/ml	4.3 ± 0.00	105
5 mg/ml, + GSH	0.48 ± 0.16	12
1.6 mg/ml, + GSH	1.18 ± 0.20	29
0.8 mg/ml, + GSH	1.72 ± 0.15	42

Fig. 1. *Frontal analysis of GSH-dependent inhibition of lipid peroxidation in rat liver soluble supernatant fraction* Rat liver soluble supernatant fraction (170 ml) was separated by frontal elution on Sephacryl S-200 equilibrated with Buffer B containing 5 mM-GSH. Fractions (1.5 ml) were collected and assayed for GSH peroxidase activity with cumene hydroperoxide (Cu-OOH) as substrate (—); GSH transferase activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (····) and the ability to inhibit lipid peroxidation of microsomes (5 mg of protein/ml) induced by 3 mM-ADP, 0.1 mM-Fe³⁺ and 0.3 mM-NADPH in Buffer B at 37°C for 5 min (----).

Fractionation of the soluble supernatant fraction by chromatography on Sephadex G-100

Concentrated soluble supernatant fraction obtained from a 1:1 homogenate was separated by

zonal elution from Sephadex G-100. Considerable dilution of the sample occurred on zonal elution, with the result that the levels of inhibition of lipid peroxidation obtained were low. Nevertheless, it

appeared that two fractions causing GSH-dependent inhibition of lipid peroxidation were eluted roughly with the Se-dependent GSH peroxidase activity and the GSH transferase activity. In order to obtain higher levels of inhibition of lipid peroxidation by fractions undiluted during separation, the technique of frontal elution was used. When soluble supernatant from a 1:3 homogenate was analysed by this technique (Fig. 1), two components which inhibited lipid peroxidation were observed as two advancing fronts at the beginning of elution and two retreating fronts at the end. The first advancing front contains the Se-dependent GSH peroxidase activity but no GSH transferase activity, and the second retreating front contains GSH transferase activity but no Se-dependent GSH peroxidase activity. The second advancing front and the first retreating front together with all the eluate in between contain both activities. Comparison of the first advancing front and the last retreating front suggests that inhibitory activity due to the GSH transferase activity is greater than that due to the Se-dependent GSH peroxidase activity, since maximal inhibition was not achieved by the total concentration of the Se-dependent fraction, but was obtained by only 30% of the total concentration of GSH transferase fraction. The above refers to soluble supernatant from phenobarbitone-treated rats in which GSH transferase activity is induced several-fold (Bass *et al.*, 1977); in soluble supernatant from normal rats the contribution of the GSH transferase fraction to inhibition of lipid peroxidation would therefore be less.

Inhibition of lipid peroxidation by purified GSH transferases

Purified GSH transferases AA, B, A and C, at concentrations of 1.8 mg/ml were added to incubations in which microsomal lipid peroxidation was initiated by ADP-Fe³⁺ plus NADPH and measured by malonaldehyde formation. GSH transferases AA and B, which contain Ya or Yc subunits and possess GSH peroxidase activity for organic peroxides, were effective inhibitors of GSH-dependent lipid peroxidation. GSH transferases A and C, which contain Yb subunits and possess little GSH peroxidase activity for organic peroxides, were not inhibitors. Since rat liver contains little GSH transferase AA (YcYc), but is abundant in GSH transferase B (a mixture of YaYa and the heterodimer YaYc), all further studies of the effects of GSH transferase were made with GSH transferase B. A reciprocal plot of data concerning the inhibition of lipid peroxidation by either soluble supernatant fraction or GSH transferase B (Tan *et al.*, 1983) showed a coincidence of ordinate intercepts. This indicates that the maxi-

mum inhibition obtainable by the soluble supernatant was also obtainable with purified GSH transferase B. GSH transferase B, at concentrations of 4 mg/ml, which is approximately physiological in normal rats (Tipping & Ketterer, 1981), causes about 90% inhibition of malonaldehyde formation (Table 1). Similar concentrations of this enzyme totally inhibit loss of microsomal phosphatidylethanolamine and the depletion of polyunsaturated fatty acyl moieties from the phosphatidylethanolamine fraction (see Table 2).

Inhibition of lipid peroxidation by GSH alone

Lipid peroxidation initiated enzymically in microsomes as described under 'Methods' was partially inhibited by GSH alone (Tan *et al.*, 1983), but the effect was not sustained (Fig. 2). The transience of the effect was not due to GSH depletion, but could be due to the formation of GSSG, which can activate lipid peroxidation in mitochondria (Hunter *et al.*, 1964). Inhibition of the initial peroxidation rate by GSH alone decreased from 45 to only 26% if the microsomes were additionally washed twice in 0.15M-Tris/HCl, pH8.0 (Morgenstern *et al.*, 1980), a procedure that removes the soluble GSH transferases (including B and AA), which are adsorbed to the membranes (Morgenstern *et al.*, 1983). The

Fig. 2. Time course of inhibition of lipid peroxidation by GSH alone and GSH plus soluble supernatant fraction. Lipid peroxidation was induced by incubating microsomes (5 mg of protein/ml), 3 mM-ADP, 0.1 mM-Fe³⁺ and 0.3 mM-NADPH (○) with 2.5 mM-GSH (●) or with 2.5 mM-GSH plus 25 mg of soluble supernatant protein/ml (□), in Buffer B at 37°C. Peroxidation was recorded (mean ± S.D.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with five other microsomal preparations gave similar results.

Table 2. Prevention by GSH transferase B of the loss of unsaturated fatty acyl groups in the phosphatidylethanolamine fraction of microsomes incubated with ADP-Fe³⁺ plus NADPH

Time (min)	Additions	[Phosphatidylethanolamine] (μmol/ml of incubation mixture)	Fatty acid	Relative fatty acid composition (%)											
				C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}	C _{20:5}	C _{22:5}	C _{22:6}			
0	Control	0.10	...	24.9	2.7	22.3	9.4	13.9	11.6	4.8	2.4	8.2			
	GSH transferase B + GSH	0.10		23.2	2.6	21.6	9.3	13.4	12.1	5.2	2.7	9.4			
20	Control	0.08		25.2	2.7	22.9	10.6	14.1	11.0	4.2	2.3	6.9			
	GSH transferase B + GSH	0.10		25.8	2.9	21.6	10.1	13.2	11.8	4.8	2.5	8.2			
60	Control	0.06		26.9	3.1	23.1	11.0	17.1	9.3	3.3	1.6	4.6			
	GSH transferase B + GSH	0.10		24.4	2.7	22.6	9.7	13.5	11.5	4.8	2.5	8.2			

Lipid peroxidation was induced by incubating microsomes (5 mg of protein/ml), 3 mM-ADP, 0.1 mM-Fe³⁺ and 0.3 mM-NADPH with 2.5 mM-GSH and GSH transferase B (4 mg/ml) in Buffer B at 37°C. The microsomal lipid was extracted and analysed by g.l.c. The data refer to one experiment; similar results were obtained when the experiment was repeated.

remaining inhibition could be due to several factors, which include the intrinsic microsomal GSH transferase (Friedberg *et al.*, 1979; Morgenstern *et al.*, 1979, 1982), the heat-labile GSH-dependent microsomal factors (Reddy *et al.*, 1982; Burk, 1982, 1983) and direct reaction of GSH with initiating radicals (Jocelyn, 1972; McNeil *et al.*, 1981; Rawley & Halliwell, 1982).

Inhibition of phospholipase A₂

It has been shown that Se-dependent GSH peroxidase and GSH transferase can utilize free fatty acid hydroperoxides as substrates, but not peroxidized phospholipids (McCay *et al.*, 1976; Gibson *et al.*, 1980; Sevanian *et al.*, 1983), and it has been suggested that these GSH peroxidases might act in the prevention of lipid peroxidation only after the hydrolytic release of free fatty acid hydroperoxides by phospholipase A₂.

The effect of inactivation of microsomal phospholipase A₂ by *p*-bromophenacyl bromide (Sevanian *et al.*, 1981) on the GSH-dependent inhibition of lipid peroxidation is given in Table 3. Under such conditions neither GSH transferase B nor the soluble supernatant exerted GSH-dependent inhibition. However, the inhibitory effect was restored if soluble, porcine pancreatic phospholipase A₂ in sufficient quantity to hydrolyse the membrane rapidly was included in the incubations. It should be noted that the control rate of lipid peroxidation was substantially decreased when phospholipase A₂ was added, an effect which can also be obtained by the addition of lysophosphatidylcholine. The effect common to both addition of lysophosphatidylcholine and extensive hydrolysis might have been either an effect on micelle formation due to the surface activity of lysophosphatides or to an increased ratio of saturated to polyunsaturated fatty acid chains in the membrane. Table 4 shows the effect of heat inactivation on microsomes. Lipid peroxidation could not be initiated enzymically and was therefore produced non-enzymically by FeCl₃/sodium ascorbate. Once again, neither GSH plus dialysed soluble supernatant nor GSH plus transferase B inhibited lipid peroxidation unless exogenous phospholipase A₂ was added.

It should be noted that both the dialysed soluble supernatant and purified GSH transferase B can cause some inhibition of lipid peroxidation in the absence of GSH in experiments in which endogenous phospholipase A₂ is added. The binding properties of GSH transferase B under the special conditions of this experiment might explain the effect obtained, but there is no certain explanation. There is no significant effect of soluble supernatant or GSH transferase B in other experiments.

Table 3. *Effect of treating microsomes with the phospholipase A₂ inhibitor p-bromophenacyl bromide (BPB) on inhibition of lipid peroxidation by soluble supernatant fraction and GSH transferase B*

Assay mixtures contained microsomes (5 mg of protein/ml), either control or BPB-treated, 3 mM-ADP, 0.1 mM-FeCl₃ and 0.3 mM-NADPH in Buffer B and were incubated at 37°C for 5 min. Where indicated, the assay mixtures contained 13 units of porcine pancreatic phospholipase A₂/ml, together with 1 mM-CaCl₂, 25 mg of soluble supernatant protein/ml, GSH transferase B (5 mg/ml) and 2.5 mM-GSH. Peroxidation was estimated (mean $\pm$ s.d.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with two other microsomal preparations gave similar results.

Incubation mixture	Peroxidation	
	(nmol of malonaldehyde/min per ml)	(% of control)
Control microsomes	5.80 $\pm$ 0.17	100
+ Soluble supernatant fraction	5.33 $\pm$ 0.00	92
+ Soluble supernatant fraction + GSH	1.45 $\pm$ 0.33	25
+ GSH transferase B	5.15 $\pm$ 0.11	89
+ GSH transferase B + GSH	1.76 $\pm$ 0.21	30
BPB-treated microsomes	4.40 $\pm$ 0.11	100
+ Soluble supernatant fraction	3.96 $\pm$ 0.06	90
+ Soluble supernatant fraction + GSH	3.92 $\pm$ 0.00	89
+ GSH transferase B	4.18 $\pm$ 0.11	95
+ GSH transferase B + GSH	3.92 $\pm$ 0.08	89
+ Phospholipase A ₂	1.80 $\pm$ 0.06	100
+ Soluble supernatant fraction	1.00 $\pm$ 0.15	55
+ Soluble supernatant fraction + GSH	0.35 $\pm$ 0.07	21
+ GSH transferase B	1.20 $\pm$ 0.00	67
+ GSH transferase B + GSH	0.56 $\pm$ 0.11	31

Table 4. *Inhibition of lipid peroxidation in heat-treated microsomes by a combination of phospholipase A₂ and soluble supernatant fraction or GSH transferase B*

Assay mixtures contained microsomes (5 mg of protein/ml), previously heated at 100°C for 10 min, 4 mM-ADP, 12 μ M-FeCl₃, 0.66 mM-sodium ascorbate and 1 mM-CaCl₂ in Buffer A and were incubated at 37°C for 5 min. Where indicated, the assay mixtures contained lysophosphatidylcholine (2 mg/ml), porcine pancreatic phospholipase A₂ (13 units/ml), 25 mg of soluble supernatant protein/ml, 5 mg of GSH transferase B/ml and 2.5 mM-GSH. Peroxidation was estimated (mean $\pm$ s.d.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with two other microsomal preparations gave similar results.

Additions	Peroxidation	
	(nmol of malonaldehyde/min per ml)	(% of control)
None	4.76 $\pm$ 0.66	100
Soluble supernatant fraction	4.99 $\pm$ 0.17	105
+ GSH	4.90 $\pm$ 0.70	103
GSH transferase B	4.38 $\pm$ 0.06	92
+ GSH	4.33 $\pm$ 0.50	91
Lysophosphatidylcholine	1.33 $\pm$ 0.38	28
Phospholipase A ₂	1.32 $\pm$ 0.16	100
+ Soluble supernatant fraction	0.72 $\pm$ 0.06	56
+ Soluble supernatant fraction + GSH	0.41 $\pm$ 0.08	30
+ GSH transferase B	0.80 $\pm$ 0.09	61
+ GSH transferase B + GSH	0.52 $\pm$ 0.00	39

Effect of phosphate on lipid peroxidation

Many studies of lipid peroxidation use a Tris buffer similar to Buffer A; however, lipid peroxidation in Tris buffer is inhibited by small amounts of phosphate buffer (see Fig. 3). Since phosphate is a normal cellular constituent, it was considered

perhaps more appropriate to use a phosphate buffer, Buffer B, rather than a Tris buffer; results obtained by using this buffer are presented in Tables 1, 2 and 4. Experiments performed under the conditions described by Gibson *et al.* (1980) with heat-inactivated microsomes suggested that

Table 5. Effect of 75 mM-phosphate buffer on lipid peroxidation in heat-treated microsomes

Assay mixtures contained microsomes (5mg of protein/ml), previously heated at 100°C for 10min, 4mM-ADP, 12 μ M-FeCl₃ and 0.66 mM-sodium ascorbate in Buffer A, and were incubated at 37°C for 5min. Where indicated, the assay mixtures included 25mg of soluble supernatant protein/ml, 2.5mM-GSH and 75mM-phosphate, pH7.5. Peroxidation was recorded (mean $\pm$ S.D.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with two other microsomal preparations gave similar results.

Additions	Peroxidation	
	(nmol of malonaldehyde/min per ml)	(% of control)
None	4.96 $\pm$ 0.16	100
Soluble supernatant fraction + 75 mM-phosphate buffer	2.38 $\pm$ 0.45	48
Soluble supernatant fraction + GSH + 75 mM-phosphate buffer	0.8 $\pm$ 0.20	16
75 mM-Phosphate buffer	2.43 $\pm$ 0.30	49
75 mM-Phosphate buffer + GSH	1.20 $\pm$ 0.18	24
Soluble supernatant fraction	4.51 $\pm$ 0.17	91
Soluble supernatant fraction + GSH	4.20 $\pm$ 0.44	84

Fig. 3. Inhibition of lipid peroxidation by phosphate buffer

Lipid peroxidation was induced by incubating microsomes (5mg of protein/ml), 3mM-ADP, 0.1mM-Fe³⁺ and 0.3mM-NADPH in Buffer A at 37°C for 5min with 0–10mM-phosphate buffer, pH7.5. Peroxidation was recorded (mean $\pm$ S.D.) as nmol of malonaldehyde released/min per ml of incubation mixture in three simultaneous incubations using one microsomal preparation; experiments with two other microsomal preparations gave similar results.

a factor may be present in soluble supernatant fraction which can inhibit lipid peroxidation in the absence of active phospholipase A₂ (see Table 4). However, it was noted that the soluble supernatant fraction used had been made 75mM with respect to phosphate, and, in the present work, identical effects on lipid peroxidation were obtained with 75mM-phosphate alone and in the absence of the soluble supernatant fraction (Table 5).

Discussion

The results show that GSH transferases B and AA (the major Se-independent GSH peroxidases) and a fraction containing the Se-dependent GSH peroxidase inhibit lipid peroxidation in untreated microsomes *in vitro*. This is in agreement with Burk

et al. (1980), who were the first to show inhibition of microsomal lipid peroxidation by the soluble GSH transferases of the rat liver. The GSH peroxidases are only part of the mechanism: microsomal phospholipase A₂ is also required, which is why the effect is not seen when microsomes are either heat-inactivated or treated with the phospholipase A₂ inhibitor *p*-bromophenacyl bromide. Phospholipase A₂ is required to release the substrates of the GSH peroxidases, which can act only on free-fatty-acyl hydroperoxides. The possibility that the consecutive action of phospholipase A₂ and GSH peroxidase might bring about inhibition of lipid peroxidation was proposed as long ago as 1965 by van Deenen and mentioned again more recently by Sevanian *et al.* (1981) and Flohé (1982), although these authors had only the Se-dependent GSH peroxidase in mind.

Recently, analogous findings to those presented here have been reported by Sevanian *et al.* (1983), who showed that lipid peroxidation induced in liposomes by lipoxygenase could be inhibited by rat liver soluble supernatant fraction only when phospholipase A₂ had been incorporated into the system.

Zimmerman (1973) has demonstrated formation of lysophosphatides during uninhibited lipid peroxidation, indicating that phospholipase A₂ is active during lipid peroxidation. A similar phenomenon has been observed, in epoxidized membranes, by Sevanian *et al.* (1981). It is assumed that the introduction of polar hydroperoxy or epoxy substituents into the hydrocarbon region of the membrane distorts the membrane at that point, either making the altered fatty acyl moiety a much better substrate for existing enzyme activity or activating the enzyme itself.

It might be thought that phospholipase A_2 , by releasing free fatty acid hydroperoxide, could alone prevent lipid peroxidation. However, the free fatty acid hydroperoxide is still relatively non-polar and would tend to remain in the membrane and retain its potential for membrane damage, were it not for the soluble GSH peroxidases, which, because of the binding affinity of their active sites followed by enzyme reaction, cause the free-fatty-acid hydroperoxides to move into the aqueous phase and undergo reduction to fatty acid alcohols.

The Se-dependent and Se-independent GSH peroxidases are not the only proteins which have been implicated in the inhibition of lipid peroxidation. Gibson *et al.* (1980) described a soluble supernatant fraction which was capable of inhibiting lipid peroxidation induced in heat-inactivated microsomes by $FeCl_3$ /sodium ascorbate. Our results suggest that the active component was not a protein, but the high phosphate concentration. The above authors also pointed out that the GSH transferases did not inhibit lipid peroxidation in this system. This is due to the absence of an active phospholipase A_2 in heat-inactivated microsomes.

Ursini *et al.* (1982) described the isolation of a peroxidation-inhibiting protein (PIP), which is a GSH peroxidase, that can utilize phospholipid hydroperoxides as a substrate. It has a similar V_{max} for the synthetic substrates cumene hydroperoxide and t-butyl hydroperoxide as have the GSH transferases B and AA, a much higher K_m for GSH (5mM), but a much lower occurrence in the soluble supernatant. PIP is not restricted to the soluble supernatant on homogenization in 0.25M-sucrose, but is found in other subcellular fractions, notably the microsomes (Ursini *et al.*, 1983). Thus it is possible that PIP, as a component of the microsomal fraction, is responsible for our observations that GSH alone partially inhibits microsomal lipid peroxidation. Furthermore, as a component of the soluble supernatant having an M_r of 23500 (Maiorini *et al.*, 1982), PIP may be responsible for a small amount of inhibitory activity trailing behind the retreating GSH transferase (M_r 45000) front in the frontal-elution analysis (see Fig. 1). A substantial effect which might be attributed to PIP was not observed.

From the present work, both Se-dependent GSH peroxidase and GSH transferases B and AA, together with phospholipase A_2 , would appear to be effective at inhibiting microsomal lipid peroxidation *in vitro*. If they play a similar role *in vivo*, recent immunohistochemical studies suggest that they are associated with different hepatic functions. For example, Se-dependent GSH peroxidase is more abundant in the periportal zone (Yoshimura *et al.*, 1980), whereas the GSH transferases

are concentrated in the centrilobular zone (Redick *et al.*, 1982). The former zone is the more oxygen-rich and associated with mitochondrial activity, whereas the latter is more concerned with xenobiotic metabolism. This suggests that the Se-dependent enzyme is largely concerned with detoxifying products of oxidizing free radicals arising from mitochondria, e.g. H_2O_2 , whereas GSH transferases B and AA are more concerned with detoxifying the products of oxidizing free radicals arising in the endoplasmic reticulum during xenobiotic metabolism.

A number of mechanisms of lipid peroxidation have been proposed (Pryor, 1976; Mead, 1976; Aust & Svingen, 1982). It has been suggested that initiation of the free-radical attack on an unsaturated fatty acid is due to an ADP-perferryl ion or iron-dioxygen complex (Svingen *et al.*, 1979; Bucher *et al.*, 1983), which results in hydrogen abstraction and the formation of a phospholipid alkyl radical. This is propagated by reaction with oxygen and the formation of hydroperoxide as shown in eqn. (1):

As shown in eqn. (2), lipid hydroperoxide can cause fresh initiation by its metal-catalysed conversion into an alkoxy radical, which then causes further hydrogen abstraction and the formation of a new phospholipid alkyl radical, as shown in eqn. (3):

Thus phospholipase A_2 , together with GSH and GSH peroxidase activity, are effective in inhibiting lipid peroxidation by preventing new initiations. The initial radical attack is not prevented, and it is possible that vitamin E terminates this step.

This work was supported by the Cancer Research Campaign, of which B.K. is a Fellow.

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