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LIPID PEROXIDATION AND ANTIOXIDANT SYSTEMS IN THE LIVER INJURY PRODUCED BY GLUTATHIONE DEPLETING AGENTS

EMILIA MAELLARO, ALESSANDRO F. CASINI,* BARBARA DEL BELLO and MARIO COMPORTI†

Istituto di Patologia Generale dell'Università di Siena, Via Laterino 8, 53100 Siena, Italy; and
* Dipartimento di Medicina Sperimentale e Scienze Biochimiche dell'Università di Perugia, Via del Giochetto, 06100 Perugia, Italy

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Abstract—The mechanisms of the liver damage produced by three glutathione (GSH) depleting agents, bromobenzene, allyl alcohol and diethylmaleate, was investigated. The change in the antioxidant systems represented by α-tocopherol (vitamin E) and ascorbic acid were studied under conditions of severe GSH depletion. With each toxin liver necrosis was accompanied by lipid peroxidation that developed only after severe depletion of GSH. The hepatic level of vitamin E was decreased whenever extensive lipid peroxidation developed. In the case of bromobenzene intoxication, vitamin E decreased before the onset of lipid peroxidation. Changes in levels of the ascorbic and dehydroascorbic acid indicated a redox cycling of vitamin C with the oxidative stress induced by all the three agents. Such a change of the redox state of vitamin C (increase of the oxidized over the reduced form) may be an index of oxidative stress preceding lipid peroxidation in the case of bromobenzene. In the other cases, such a change is likely to be a consequence of lipid peroxidation. Experiments carried out with vitamin E deficient or supplemented diets indicated that the pathological phenomena occurring as a consequence of GSH depletion depend on hepatic levels of vitamin E. In vitamin E deficient animals, lipid peroxidation and liver necrosis appeared earlier than in animals fed the control diet. Animals fed a vitamin E supplemented diet had an hepatic vitamin E level double that obtained with a commercial pellet diet. In such animals, bromobenzene and allyl alcohol had only limited toxicity and diethylmaleate none in spite of comparable hepatic GSH depletion. Thus, vitamin E may largely modulate the expression of the toxicity by GSH depleting agents.

A number of hepatotoxins are converted to electrophilic metabolites that readily conjugate with hepatic glutathione (GSH‡) to produce extensive GSH depletion followed by the appearance of cellular damage. We have studied the liver injury produced in intact mice by three GSH depleting agents that are metabolized differently by the liver; (i) bromobenzene, that is metabolized by the microsomal monooxygenase system [1–3]; (ii) allyl alcohol that is metabolized by the cytosolic enzyme alcohol dehydrogenase to acrolein [4, 5] and then by aldehyde dehydrogenase to acrylic acid [6]; (iii) and diethylmaleate which is mainly conjugated with GSH by GSH-transferases [7].

With each of these toxins, hepatic GSH decreases. After the GSH concentration has reached a critical value, lipid peroxidation and liver necrosis develop [8–11]. A similar course of events has been described also in the case of acetaminophen intoxication [12]. In the case of bromobenzene, treatment of the intoxicated animals with the antioxidant Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a homolog of vitamin E), or with the iron chelator desferrioxamine (DFO), completely prevents both lipid peroxidation and liver necrosis [8, 9]. The extent of the covalent binding of bromobenzene metabolites to liver protein was not affected by Trolox C [8]. A similar result was obtained [13] in cultured hepatocytes treated with bromobenzene and the antioxidant *N,N'*-diphenyl-*p*-phenylenediamine (DPPD). In addition DFO has been shown to suppress lipid peroxidation and liver injury in allyl alcohol-intoxicated mice [14]. Thus, lipid peroxidation seems to be a factor in the pathogenetic mechanisms of liver necrosis produced by these GSH depleting agents.

The endogenous defences against the peroxidation of membrane lipids remain an area of continuous interest. In addition to α-tocopherol (vitamin E), ascorbic acid (vitamin C) has also been implicated in such defences. Vitamin E is consumed during lipid peroxidation *in vitro* [15–18]. The role of vitamin C is less clear [19, 20]. Other antioxidant systems are those in which GSH acts as an hydrogen donor [21–24]. In the present report we investigated the changes in vitamin E and vitamin C during the development of the liver injury induced by bromobenzene, diethylmaleate and allyl alcohol. Such changes were followed both during the development of lipid peroxidation and prior to the onset of it. The results support the view for a redox cycling of vitamin C accompanying lipid peroxidation and vitamin E consumption in *in vivo* conditions. This conclusion is strengthened by the results obtained with studies carried out in animals maintained on a vitamin E-deficient diet or a vitamin E-enriched diet.

† To whom correspondence should be sent.

‡ Abbreviations used: GSH, glutathione; DFO, desferrioxamine; SGPT, serum glutamate-pyruvate transaminase; MDA, malonic dialdehyde; HPLC, high-pressure liquid chromatography.

Table 1. Fatty acid composition of liver phospholipids of mice maintained on a vitamin E deficient diet or the same diet supplemented with 30 or 65 mg of vitamin E/kg

	% of total fatty acids		
Fatty acid	Vitamin E deficiency	Vitamin E supplementation (30 mg/kg diet)	Vitamin E supplementation (65 mg/kg diet)
16:0	17.1 ± 1.4	18.4 ± 1.0	16.7 ± 2.1
16:1	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.3
18:0	14.4 ± 0.8	14.6 ± 0.5	15.4 ± 0.2
18:1	15.0 ± 0.4	17.0 ± 0.5	15.9 ± 0.5
18:2	11.8 ± 0.7	10.9 ± 0.4	12.9 ± 0.4
20:4	28.4 ± 1.6	27.4 ± 1.0	27.3 ± 1.1
22:6	11.4 ± 1.0	10.2 ± 0.8	10.1 ± 1.0

For the vitamin E deficient or supplemented diets, see Materials and Methods. Values reported are means ± SE obtained from three animals.

MATERIALS AND METHODS

Male NMRI albino mice (Charles River Italia, Como, Italy) weighing 20–30 g were used. As reported in previous studies [8], mice are more susceptible to bromobenzene and diethylmaleate-intoxication than are rats. The animals were maintained on different diets. Some of the animals were maintained on a complete pellet diet (Altromin-Rieper, Bolzano, Italy; vitamin E addition 75 mg/kg, company data), hereafter referred to as the standard laboratory diet. The remaining animals were subdivided into three groups: one group was maintained, since weaning, on a vitamin E-deficient diet (prepared by Ditta Piccioni, Brescia, Italy, as described by Evans and Burr [25], modified as described by Bacharach and Allchorne [26]; the selenium content of the diet was 0.11–0.12 ppm); the other two groups were maintained on the same diet (basal diet) supplemented with 30 or 65 mg of vitamin E/kg, respectively.

Vitamin E deficiency or vitamin E supplementation of the diet had no effects on the fatty acid composition of liver phospholipids (Table 1). In particular the content of polyunsaturated fatty acids was virtually the same in the three groups of animals.

The animals were starved overnight (16 hr in all) before intoxication. Starvation decreased hepatic GSH by about 50% as compared with that in fed animals and increased the frequency of liver necrosis.

Bromobenzene (C. Erba, Milan, Italy) mixed with two volumes of mineral oil was administered intragastrically under light ether anesthesia at the dose of 13 mmol/kg body wt. Control mice received mineral oil alone. Allyl alcohol (C. Erba, Milan, Italy), dissolved in saline solution (10% v/v), was given intraperitoneally at the dose of 1.5 mmol/kg body wt. Control mice received saline only. Diethylmaleate (Fluka AG, Buchs, Switzerland) was administered undiluted at the dose of 12 mmol/kg body wt by gastric intubation. All the animals were starved after intoxication.

Hepatic GSH was measured as acid-soluble -SH groups according to Sedlak and Lindsay [27].

Liver damage was assessed by measuring the serum glutamate-pyruvate transaminase (SGPT) activity (optimized UV enzymatic method, C. Erba).

Lipid peroxidation was measured in the whole liver as tissue content of malonic dialdehyde (MDA) as previously reported [28]. It was ascertained in preliminary experiments that the method measuring the tissue content of MDA correlated with the other methods generally run in this laboratory to detect lipid peroxidation: in particular with the measurement of the diene conjugation absorption in cellular phospholipids, as done by Casini *et al.* [13], and with the evaluation of the amount of carbonyl functions originating from the peroxidative breakdown of unsaturated fatty acids in cellular phospholipids as done by Benedetti *et al.* [29].

Hepatic α-tocopherol was extracted and measured by high-pressure liquid chromatography (HPLC) as reported by Burton *et al.* [30]. According to this method α-tocopherol can be revealed by either UV detection or fluorescence detection. This was true for measurement of tocopherol in normal livers (Fig. 1A). However, when tocopherol was determined in livers showing a high level of lipid peroxidation (as measured by MDA content), UV detection showed a number of peaks (Fig. 1B) (with a major peak in some instances, Fig. 1B) which were hardly distinguishable from that of α-tocopherol, due to similar retention time values. The addition of standard α-tocopherol to the samples (Fig. 1C) revealed that none of these peaks was α-tocopherol. On the other hand, fluorescence detection showed the peak of α-tocopherol in both the absence (Fig. 1A) and the presence (Fig. 1B) of lipid peroxidation.

Hepatic ascorbic and dehydroascorbic acids were measured by HPLC in metaphosphoric acid extracts as reported by Garcia-Castineiras *et al.* [31]. This method, that is based on measurement of the oxidized form of ascorbic acid and measurement of the reduced form by difference after oxidation of the sample, was in our experience a suitable method to avoid artifactual oxidation of the reduced form. Protein determination was performed according to Lowry *et al.* [32].

The fatty acid composition of liver phospholipids was determined following acetone-precipitation of phospholipids according to Borgström [33]. Fatty acid methyl esters were prepared and analysed by gas liquid chromatography as previously reported [34].

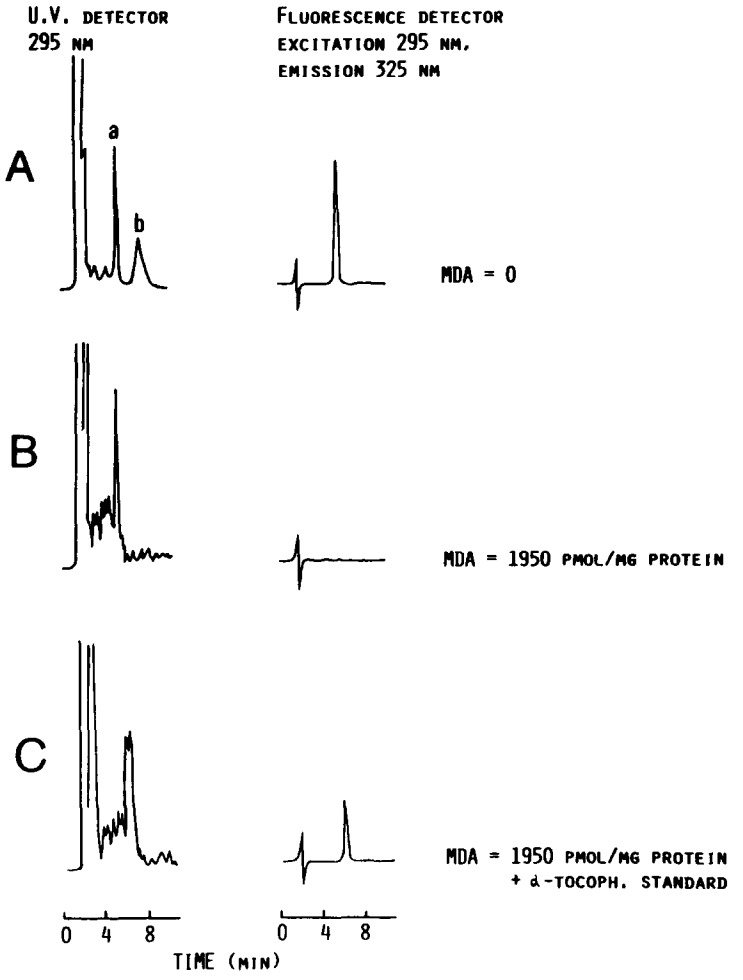

Fig. 1. High-pressure liquid chromatography (HPLC) determination of α-tocopherol by both UV detection and fluorescence detection. (A) Determination of α-tocopherol in the liver of a control animal (malonic dialdehyde, MDA, content of the liver = 0). Both UV and fluorescence detection reveals α-tocopherol (peak a; peak b, unknown). (B) Determination of α-tocopherol in the liver of a bromobenzene-intoxicated animal (MDA content of the liver = 1950 pmol/mg protein). As can be seen, the UV detection shows a number of peaks together with a major peak which is hardly distinguishable from α-tocopherol due to similar retention time values. However the fluorescence detection does not show any peak in the presence of high levels of lipid peroxidation. (C) Same sample as that shown in (B) injected together with a suitable amount of standard α-tocopherol. As can be seen two unresolved peaks are visible under UV detection. The fluorescence detection shows α-tocopherol only.

RESULTS

Bromobenzene

Mice maintained on the standard laboratory diet and intoxicated with bromobenzene showed a rapid GSH depletion that was nearly maximal at 3 hr (Table 2). Lipid peroxidation and liver necrosis were detected after 15 hr. Both were increased in severity at 18 hr. The hepatic content of vitamin E was unchanged during the first 9 hr of intoxication. A significant decrease was observed at 12 hr, a time prior to the development of lipid peroxidation. A further decrease occurred as lipid peroxidation increased. Ascorbic acid was significantly increased at 3 hr and then steadily declined to reach a value 50% of the control one at 18 hr. Dehydroascorbic acid was increased throughout the intoxication period and prior to the appearance of lipid peroxidation.

Diethylmaleate

The intoxication of mice with diethylmaleate resulted in a rapid depletion of GSH (Table 3) with some return of the GSH content at the later times. Lipid peroxidation and liver necrosis developed at 15–20 hr and only in those animals (about 40%) in which the level of GSH remained low. Vitamin E was unchanged until lipid peroxidation was well developed. Evaluation of the redox state of vitamin C showed that the increase in the oxidized form (dehydroascorbic acid) after 20 hr quantitatively reflected the decrease in the reduced form (Table 3).

Table 2. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after bromobenzene intoxication of mice maintained on the standard laboratory diet

Time after intoxication (bromobenzene)	0 time	3 hr	9 hr	12 hr	15 hr	18 hr
GSH	24.3 ± 1.7	3.7 ± 0.2	2.1 ± 0.1	2.2 ± 0.2	2.4 ± 0.3	1.9 ± 0.3
(nmol/mg protein)	(20)	(7)	(6)	(15)	(14)	(28)
SGPT	46 ± 5	48 ± 30	35 ± 6	63 ± 15	2578 ± 1389	4669 ± 1545
(units/L)	(20)	(3)	(6)	(12)	(14)	(28)
MDA	—	0	0	3 ± 2	189 ± 85	1097 ± 406
(pmol/mg protein)		(3)	(6)	(15)	(14)	(11)
Vitamin E	122 ± 6	149 ± 35	108 ± 2	86 ± 22*	67 ± 16‡	45 ± 16‡
(pmol/mg protein)	(20)	(7)	(6)	(11)	(14)	(11)
Ascorbic acid	7.9 ± 0.4	11.7 ± 0.2‡	ND	8.5 ± 0.7	6.6 ± 1.2	4.0 ± 0.8‡
(nmol/mg protein)	(20)	(7)		(11)	(14)	(16)
Dehydroascorbic acid	0.37 ± 0.07	1.02 ± 0.21†	ND	1.55 ± 0.24‡	1.32 ± 0.16‡	0.59 ± 0.38
(nmol/mg protein)	(19)	(3)		(10)	(14)	(5)

For the standard laboratory diet, see Materials and Methods. Bromobenzene was given by gastric intubation at the dose of 13 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.
*–‡ Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.005$; ‡ $P < 0.001$.
ND, not determined.

Table 3. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after diethylmaleate intoxication of mice maintained on the standard laboratory diet

Time after intoxication (diethylmaleate)	0 time	1 hr	2 hr	15 hr	20 hr
GSH	23.7 ± 1.5	2.5 ± 0.1	2.6 ± 0.6	6.9 ± 1.9	5.1 ± 1.3
(nmol/mg protein)	(5)	(3)	(3)	(8)	(10)
SGPT	29 ± 6	41 ± 16	59 ± 16	233 ± 106	1791 ± 692
(units/L)	(4)	(3)	(3)	(8)	(10)
MDA	—	1.3 ± 1.3	3 ± 3	86 + 49	511 ± 214
(pmol/mg protein)		(3)	(3)	(8)	(10)
Vitamin E	91 ± 9	115 ± 17	137 ± 24	75 ± 7	46 ± 11†
(pmol/mg protein)	(5)	(3)	(3)	(8)	(10)
Ascorbic acid	5.5 ± 1.2	4.9 ± 0.3	5.0 ± 0.9	ND	3.6 ± 0.9
(nmol/mg protein)	(3)	(3)	(3)		(10)
Dehydroascorbic acid	0.75 ± 0.04	0.88 ± 0.04	0.86 ± 0.06	1.12 ± 0.09*	2.58 ± 0.34†
(nmol/mg protein)	(3)	(3)	(3)	(8)	(10)

For the standard laboratory diet, see Materials and Methods. Diethylmaleate was given by gastric intubation at the dose of 12 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.
*† Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.025$.
ND, not determined.

Allyl alcohol

Table 4 shows the results obtained with allyl alcohol. GSH depletion was maximal at 15–30 min, whereas lipid peroxidation and liver necrosis occurred at 2–4 hr. Vitamin E was decreased at 4 hr. By contrast ascorbic acid was significantly decreased as early as 30 min. Dehydroascorbic acid was increased only at 4 hr.

Experiments carried out in mice maintained on a diet containing different levels of vitamin E

Intoxications with the three GSH depleting agents were performed in animals maintained on either a vitamin E deficient diet or the same diet supplemented with either 30 or 65 mg of vitamin E/kg. After feeding these different dietary regimens for 40 days, the hepatic level of α-tocopherol was 4–5, 90–130 and 230–300 pmol/mg protein with the vitamin E deficient diet (basal diet), the basal diet supplemented with 30 mg of vitamin E/kg and the basal diet supplemented with 65 mg of vitamin E/kg, respectively. The amounts of vitamin E added to the diet (30 or 65 mg/kg) achieved hepatic α-tocopherol concentrations equal to or double that obtained with the standard laboratory diet.

In mice maintained on the vitamin E deficient diet, bromobenzene intoxication caused (Table 5) the development of lipid peroxidation and liver necrosis much earlier (9 hr vs 15–18 hr) and more severely (SGPT 14,570 vs 4669 units/L) than in mice fed

Table 4. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after allyl alcohol intoxication of mice maintained on the standard laboratory diet

Time after intoxication (allyl alcohol)	0 time	30 min	2 hr	4 hr
GSH	22.3 ± 0.8	2.4 ± 0.2	3.3 ± 0.4	6.3 ± 1.0
(nmol/mg protein)	(19)	(18)	(24)	(19)
SGPT	36 ± 7	119 ± 39	591 ± 206	1639 ± 310
(units/L)	(17)	(17)	(24)	(26)
MDA	—	12 ± 2	55 ± 16	225 ± 63
(pmol/mg protein)		(17)	(27)	(27)
Vitamin E	63 ± 4	63 ± 2	64 ± 7	44 ± 6*
(pmol/mg protein)	(12)	(9)	(10)	(23)
Ascorbic acid	5.7 ± 0.4	4.0 ± 0.4†	3.7 ± 0.4‡	2.7 ± 0.2§
(nmol/mg protein)	(16)	(18)	(24)	(15)
Dehydroascorbic acid	0.66 ± 0.06	0.69 ± 0.05	0.74 ± 0.05	0.93 ± 0.09*
(nmol/mg protein)	(16)	(18)	(24)	(14)

For the standard laboratory diet, see Materials and Methods. Allyl alcohol was given intraperitoneally at the dose of 1.5 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.

*–§ Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

Table 5. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after bromobenzene intoxication of mice maintained on either a vitamin E deficient diet or the same diet supplemented with 65 mg of vitamin E/kg

Time after intoxication (bromobenzene)	Vitamin E deficient mice		Vitamin E supplemented mice		
	0 time	9 hr	0 time	9 hr	21 hr
Vitamin E	5.6 ± 0.9	traces	228 ± 24	299 ± 47	ND
(pmol/mg protein)	(5)	(8)	(4)	(8)	
GSH	26.5 ± 1.7	3.3 ± 0.3	24.7 ± 1.7	3.7 ± 0.4	2.7 ± 0.2
(nmol/mg protein)	(6)	(12)	(4)	(8)	(7)
SGPT	32 ± 7	14,570 ± 2158	31 ± 8	174 ± 38	286 ± 61
(units/L)	(5)	(11)	(4)	(7)	(7)
MDA	—	880 ± 81	—	24 ± 6	30 ± 7
(pmol/mg protein)		(10)		(8)	(7)
Ascorbic acid	8.0 ± 1.4	3.5 ± 1.0*	8.0 ± 0.5	9.2 ± 1.7	ND
(nmol/mg protein)	(4)	(9)	(4)	(7)	
Dehydroascorbic acid	0.59 ± 0.06	1.70 ± 0.20‡	0.64 ± 0.06	0.88 ± 0.05†	ND
(nmol/mg protein)	(4)	(9)	(4)	(7)	

For the vitamin E deficient or vitamin E supplemented diet, see Materials and Methods. Bromobenzene was given by gastric intubation at the dose of 13 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.

*–‡ Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$.

ND, not determined.

standard diet. With the vitamin E deficient diet, ascorbic acid decreased and dehydroascorbic acid increased with the early onset of lipid peroxidation.

Interestingly, only minor effects of bromobenzene intoxication were seen (Table 5) in animals fed the same deficient diet supplemented with 65 mg of vitamin E/kg, in spite of a comparable hepatic GSH depletion. Even at 21 hr liver necrosis and lipid peroxidation were very low in this group of animals. The change in the redox state of vitamin C was also minimal.

The effects of vitamin E depletion could be attributed specifically to the change in vitamin E content of the livers. In fact, when diet was supplemented with vitamin E (30 mg/kg) to achieve an hepatic content identical to that obtained with the standard diet, bromobenzene intoxication produced similar results (Table 6) as those with the standard diet (Table 2).

The most striking effects of the status of the animals with respect to dietary vitamin E were seen after diethylmaleate administration. In animals fed on the standard laboratory diet lipid peroxidation and liver necrosis occurred at 20 hr only (Table 3). By contrast, in vitamin E-deficient mice both phenomena occurred as early as 10 hr (Table 7). Again dehydroascorbic acid was increased. Importantly no lipid peroxidation,

Table 6. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after bromobenzene intoxication of mice maintained on a vitamin E deficient diet supplemented with 30 mg of vitamin E/kg

Time after intoxication (bromobenzene)	0 time	9 hr	15–17 hr
Vitamin E	111 ± 11	84 ± 14	49 ± 10‡
(pmol/mg protein)	(5)	(6)	(12)
GSH	24.2 ± 1.7	4.3 ± 0.4	1.7 ± 0.2
(nmol/mg protein)	(8)	(6)	(12)
SGPT	21 ± 3	24 ± 4	3718 ± 1274
(units/L)	(6)	(6)	(12)
MDA	—	11 ± 3	608 ± 136
(pmol/mg protein)		(6)	(12)
Ascorbic acid	5.8 ± 0.4	7.3 ± 0.4*	3.3 ± 0.8†
(nmol/mg protein)	(8)	(6)	(12)
Dehydroascorbic acid			
Dehydroascorbic acid	0.59 ± 0.11	0.67 ± 0.05	1.29 ± 0.31
(nmol/mg protein)	(8)	(6)	(12)

For the vitamin E deficient diet supplemented with vitamin E, see Materials and Methods. Bromobenzene was given by gastric intubation at the dose of 13 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.

*–‡ Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.025$; ‡ $P < 0.005$.

Table 7. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after diethylmaleate intoxication of mice maintained on either a vitamin E deficient diet or the same diet supplemented with 65 mg of vitamin E/kg

Time after intoxication (diethylmaleate)	Vitamin E depleted mice		Vitamin E supplemented mice	
	0 time	10 hr	0 time	10 hr
Vitamin E	4.1 ± 0.9	1.6 ± 1.0	270 ± 37	297 ± 23
(pmol/mg protein)	(5)	(11)	(6)	(9)
GSH	33.0 ± 3.4	4.5 ± 0.7	27.0 ± 1.7	5.0 ± 0.5
(nmol/mg protein)	(5)	(11)	(6)	(9)
SGPT	39 ± 10	3622 ± 1264	32 ± 5	45 ± 11
(units/L)	(5)	(11)	(6)	(9)
MDA	—	461 ± 150	—	4 ± 2
(pmol/mg protein)		(11)		(9)
Ascorbic acid	4.2 ± 0.3	4.1 ± 0.7	5.4 ± 0.7	6.2 ± 0.6
(nmol/mg protein)	(5)	(11)	(6)	(8)
Dehydroascorbic acid	0.60 ± 0.09	1.29 ± 0.20*	0.62 ± 0.07	0.78 ± 0.08
(nmol/mg protein)	(5)	(11)	(6)	(8)

For the vitamin E deficient or vitamin E supplemented diet, see Materials and Methods. Diethylmaleate was given by gastric intubation at the dose of 12 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.

* Significantly different from the 0 time value: $P < 0.01$.

no liver necrosis and no change in the redox state of ascorbic acid occurred in animals fed vitamin E supplemented (65 mg/kg) diet (Table 7), in spite of a comparable hepatic GSH depletion.

Similar results were obtained with allyl alcohol (Table 8). In animals fed the vitamin E-deficient diet, lipid peroxidation and liver necrosis were evident at 1 hr; analogous findings have been described by Jaeschke *et al.* [14] with a low vitamin E diet. Both lipid peroxidation and necrosis were minimal in animals fed the same diet supplemented with 65 mg of vitamin E (Table 8). After 2 hr lipid peroxidation and liver necrosis remained very low in this group of animals. Ascorbic acid was decreased and dehydroascorbic acid was increased in vitamin E depleted mice. These changes were not prevented by the vitamin E supplemented diet.

DISCUSSION

In the present study lipid peroxidation and liver

Table 8. Time-course of hepatic glutathione (GSH) depletion, liver necrosis (SGPT), lipid peroxidation (hepatic content of malonic dialdehyde, MDA) and change in hepatic α-tocopherol (vitamin E), ascorbic acid and dehydroascorbic acid content after allyl alcohol intoxication of mice maintained on either a vitamin E deficient diet or the same diet supplemented with 65 mg of vitamin E/kg

Time after intoxication (allyl alcohol)	Vitamin E depleted mice		Vitamin E supplemented mice		
	0 time	1 hr	0 time	1 hr	2 hr
Vitamin E (pmol/mg protein)	4.2 ± 0.7 (4)	0.5 ± 0.3 (9)	307 ± 36 (9)	322 ± 50 (8)	356 ± 69 (4)
GSH (nmol/mg protein)	26.7 ± 3.1 (4)	2.8 ± 0.2 (9)	23.9 ± 2.6 (4)	6.6 ± 0.9 (9)	6.4 ± 0.8 (4)
SGPT (units/L)	40 ± 11 (4)	1322 ± 402 (9)	31 ± 4 (4)	67 ± 19 (9)	284 ± 194§ (4)
MDA (pmol/mg protein)	—	499 ± 54 (9)	—	16 ± 9 (9)	5 ± 1 (4)
Ascorbic acid (nmol/mg protein)	4.3 ± 0.4 (4)	3.5 ± 0.2‡ (9)	6.0 ± 1.0 (4)	3.9 ± 0.3* (9)	3.5 ± 0.4* (4)
Dehydroascorbic acid (nmol/mg protein)	0.50 ± 0.07 (4)	0.75 ± 0.03† (9)	0.54 ± 0.03 (4)	0.68 ± 0.06 (9)	0.70 ± 0.10 (4)

For the vitamin E deficient or vitamin E supplemented diet, see Materials and Methods. Allyl alcohol was given intraperitoneally at the dose of 1.5 mmol/kg body wt. Results are given as means ± SE. The number of animals is reported in parentheses.

*–‡ Significantly different from the 0 time value: * $P < 0.05$; † $P < 0.025$; ‡ $P < 0.005$.

§ SGPT values were normal in two cases and moderately elevated in the other two cases.

necrosis were induced by three different GSH-depleting agents, bromobenzene, diethylmaleate and allyl alcohol. The conclusion that lipid peroxidation is an important pathogenetic mechanism of liver necrosis was supported by the studies carried out with vitamin E-deficient animals. In these animals, the development of liver necrosis was accelerated and its extent greater. Lipid peroxidation also developed much earlier as compared to the animals fed on the standard laboratory diet. In addition, supplementation of the diet with excess of vitamin E prevented both lipid peroxidation and necrosis. However, some caution must be exercised in necessarily attributing this protection by vitamin E to the inhibition of lipid peroxidation. Whereas vitamin E supplementation did not modify the rate and the extent of GSH depletion, more subtle effects on the metabolism of bromobenzene and allyl alcohol cannot be excluded. It should be noted, however, that administration of a derivative of α-tocopherol (Trolox C) did not affect the covalent binding of bromobenzene metabolites to cellular proteins at the same time that it prevented liver necrosis [8].

These three models of oxidative liver cell death were used to assess the accompanying changes in the two major endogenous antioxidants, vitamins E and C. With each of the toxins studied vitamin E was consumed in parallel with lipid peroxidation. Whereas vitamin E consumption has been documented in *in vitro* systems [15–18], vitamin E depletion has not been shown to accompany lipid peroxidation in intact animals, other than in carbon tetrachloride-intoxicated animals [35]. The demonstration here of the significant loss of vitamin E upon lipid peroxidation supports the hypothesis that vitamin E is in fact an important endogenous antioxidant in the intact animal. Furthermore, in bromobenzene intoxication vitamin E decreased before the onset of lipid peroxidation. This was true in both the animals fed the standard laboratory diet (Table 2) and those fed the basal diet supplemented with 30 mg of vitamin E/kg (Table 6). The same result was also observed in a group of animals fed the basal diet plus 15 mg of vitamin E/kg (hepatic vitamin E level in non-treated controls, 65 ± 6.5 pmol/mg protein) and equally intoxicated with bromobenzene (data not reported). Thus, it seems that mechanisms are elicited whereby vitamin E is consumed in the membranes prior to the appearance of lipid peroxidation.

The data presented in this report are also the first demonstration of the redox cycling of vitamin C with an oxidative stress in an intact animal. Such redox cycling was most clearly evident with diethylmaleate (Table 3). In this situation, an accumulation of oxidized vitamin C reflected quantitatively the decrease of the reduced form. This change again occurred in parallel with peroxidation of cellular lipids. In the case of allyl alcohol, there was a greater loss of the reduced form than could be accounted for by the accumulation of the oxidized one. This most likely reflects the fact that acrolein, the proximate metabolite of allyl alcohol, can react with reduced vitamin C [36, 37]. With bromobenzene, there was an early increase in reduced vitamin C accompanied by a significant and more pronounced increase in the oxidized form. In addition to this redox cycling of vitamin C, an early induction of the synthesis of ascorbic acid can account for these results. Of particular interest is the fact that in the case of bromobenzene such changes in the redox state of vitamin C were evident well before the onset of lipid peroxidation. This change in the reduced/oxidized ascorbic acid ratio is in agreement with the early decrease in vitamin E and can be regarded as an index of oxidative stress that precedes and signals the appearance of lipid peroxidation. With diethylmaleate and allyl alcohol the change in the redox state of vitamin C and the decrease in vitamin E appears to be the consequence

of uncontrolled lipid peroxidation. Differences in the extent of oxidative stress induced by the three toxins can be invoked to explain this diversity.

In summary, the present study continues to argue for a casual relationship between GSH-depletion, induction of lipid peroxidation and appearance of liver cell necrosis. In addition the data presented suggest that vitamins E and C play an important role in the intact animal in the defence against this sequence of events. In particular, vitamin E appears to affect to a great extent the expression of the hepatotoxicity of GSH-depleting agents. In fact, in the animals fed the vitamin E rich diet (65 mg/kg), the three toxins had only minor effects in spite of a comparable GSH depletion. Thus, even under conditions of extreme GSH depletion, an hepatic level of vitamin E approximately double that obtained with the commercial standard diet, can prevent the pathologic phenomena occurring in the liver cell.

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