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ALLYL ALCOHOL-INDUCED HEMOLYSIS AND ITS RELATION TO IRON RELEASE AND LIPID PEROXIDATION

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Abstract—Allyl alcohol administration to starved mice produced, along with liver necrosis, a high incidence (about 50%) of hemolysis. A marked decrease in erythrocyte glutathione (GSH) was seen in all the intoxicated animals. Such a decrease was significantly higher in the animals showing hemolysis. In these animals a substantial amount of malonic dialdehyde (MDA) was detected in plasma and a marked decrease in arachidonic and docosahexaenoic acids was found in erythrocyte phospholipids. These data suggest that the allyl alcohol-induced hemolysis is mediated by lipid peroxidation.

In vitro studies have shown that the addition of acrolein to mouse erythrocytes produces a dramatic GSH depletion, which is followed by the appearance of lipid peroxidation and, after an additional 30 min of incubation, by the development of hemolysis. Prevention of lipid peroxidation by an antioxidant (Trolox C) or an iron chelator (desferrioxamine, DFO), prevented hemolysis even if the erythrocyte GSH level was dramatically decreased. *In vitro*, allyl alcohol and acrylic acid were ineffective in inducing GSH depletion, lipid peroxidation and hemolysis. Studies of possible induction of lipid peroxidation in erythrocytes showed that a progressive increase in "free" (desferal chelatable) iron occurs in the erythrocytes during the incubation with acrolein. It seems, therefore, that a release of iron from iron-containing complexes occurs in acrolein-treated erythrocytes and that such "free" iron promotes lipid peroxidation.

It is well known that allyl alcohol produces periportal necrosis of the liver in rats and mice [1]. Allyl alcohol is metabolized by the cytosolic enzyme alcohol dehydrogenase to acrolein [2, 3]. The latter can be further metabolized to acrylic acid but can also react rapidly with cellular molecules [4]. Because of its high reactivity, acrolein is considered the most important metabolite responsible for damage induced by allyl alcohol in liver and other tissues. In fact acrolein, which is the most toxic member of the class of 2-alkenals [5, 6], is a powerful electrophile that reacts even spontaneously with nucleophiles such as sulphydryl groups [7]. The reaction products are adducts resulting from the addition of the terminal ethylenic carbon atom to the -SH compound by a thioether linkage [5–8]. Cellular glutathione (GSH)† is primarily involved in the reaction, and the result is a dramatic loss of GSH stores [5, 9–11].

Allyl alcohol is toxic not only for the liver cell, but also for other types of cells, such as the renal epithelial cells [12] and the pancreatic acinar cells [13], in which alcohol dehydrogenase is present [12, 13] although at a lower level as compared to the liver cells. The possibility has also been considered [14] that allyl alcohol metabolites, released from the liver cell, undergo a transformation to the toxic compound acrolein which would produce damage to extrahepatic tissues.

During the course of studies concerned with the mechanisms of allyl alcohol-induced liver injury, we have observed that a severe hemolysis appears in about 50% of the intoxicated mice. Since it has been demonstrated [15–17] that lipid peroxidation develops in the liver following allyl alcohol intoxication, as in the case of intoxications with other GSH-depleting agents [18], we investigated whether lipid peroxidation occurs in the membranes of erythrocytes of allyl alcohol-treated mice and whether acrolein reproduces the effects of the intoxication in *in vitro* systems. Possible mechanisms of induction of lipid peroxidation were also investigated. The possibility is shown that an iron release from iron stores occurs in GSH depleted cells. Such iron may play a role in the induction of lipid peroxidation.

MATERIALS AND METHODS

Desferrioxamine (Desferal®, DFO) and Trolox C (6 - hydroxy - 2,5,7,8 - tetramethyl - chroman - 2 - carboxylic acid) were kindly supplied by Ciba Geigy (Basel, Switzerland) and Hoffmann La Roche Inc. (Basel, Switzerland), respectively. The solvents used for high-pressure liquid chromatography (HPLC) analysis were of HPLC grade. All other chemicals were of analytical grade.

Male NMRI albino mice (Nossan, Correzzana, Milan, Italy) weighing 25–35 g and maintained on a pellet diet (Nossan) were used. The animals were starved 24 hr before receiving allyl alcohol (1.5 mmol/kg body wt, i.p.) dissolved in saline, or an equivalent volume of saline. Starvation decreases the hepatic GSH stores and renders the animals more susceptible to the toxic effects of GSH-depleting agents. The animals were sacrificed 1–2 hr after the intoxication. Blood was withdrawn from the abdominal aorta under ether anaesthesia and heparinized.

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† Abbreviations used: GSH, glutathione; DFO, desferrioxamine; HPLC, high-pressure liquid chromatography; MDA, malonic dialdehyde; FO, ferrioxamine.

An aliquot was used for GSH determination according to Beutler *et al.* [19]. Another aliquot was centrifuged (800 *g* for 10 min) and the malonic dialdehyde (MDA) content of plasma was determined after protein precipitation with trichloroacetic acid (final concentration 5%) and the addition of thiobarbituric acid [20]. The percentage of hemolysis was determined by measuring the optical density at 540 nm of the diluted plasma and by comparing this value with the optical density of diluted blood completely hemolyzed by the addition of a hypotonic solution of EDTA (2.7 mM).

In the experiments in which the fatty acid composition of erythrocyte phospholipids was determined, washed erythrocytes were extracted with a chloroform:methanol (2:1, v/v) mixture according to Folch *et al.* [21]. Phospholipids were precipitated from the total lipid extract according to Borgström [22]. Fatty acid methyl esters were prepared and analyzed by gas–liquid chromatography as previously reported [23].

In the *in vitro* studies, iron contamination was removed from the buffers used as follows: DFO was added to the buffer at a concentration of 10 μM. The buffers were chromatographed on a column of 15 g of silicic acid (Kieselgel 100, 0.063–0.2 mm, Merck, Darmstadt, West Germany) which had been washed with 200 ml of 1 N HCl and 300 ml of H_2O. Ferrioxamine (FO) and the excess of DFO were retained by silicic acid as assessed by the subsequent elution and detection by HPLC as indicated below.

Washed erythrocytes from starved, non-intoxicated mice, were incubated as a 50% (v/v) suspension in 0.123 M NaCl, 0.028 M K–Na phosphate buffer, pH 7.4. Acrolein was added in a small volume of buffer at the final concentrations of 6 or 3 mM. Glutathione, MDA and hemolysis were determined as reported above.

In the experiments designed for the determination of "free iron" (DFO-chelatable) the erythrocytes were incubated in 0.1 M KCl, 0.05 M Tris–maleate buffer, pH 7.4 in presence of 50 μM DFO added 15 min after acrolein addition. At the end of the incubation, erythrocytes were hemolyzed by addition of water (1 vol.) and sonication of the sample in a ultrasound bath. After centrifugation (10,000 *g* for 15 min) the supernatant was ultrafiltered in a ultrafiltration cone (Centriflo® CF25, Amicon). "Free iron" was determined as a DFO-iron complex (FO) by HPLC essentially as reported by Kruck *et al.* [24] with a number of modifications. The excess DFO in the samples (that is, DFO that had not reacted with "free iron") was removed by silicic acid column chromatography. Polypropylene columns (0.8 × 4 cm, Bio-Rad Laboratories, Richmond, CA) containing 250 mg of Kieselgel 60 (Merck, 0.015–0.04 mm) previously washed with 2 ml of 1 N HCl and 3 ml of H_2O, were used. After the sample was applied on the column, 1.5 ml of H_2O were added to remove the buffer salts. Then 1 ml of methanol was introduced into the column to dry the silica gel. Unreacted DFO was eluted with 5 ml of a solvent mixture made up by methanol:acetonitrile:glacial acetic acid:33% ammonia (50:50:0.2:0.2, by vol.). Under these conditions DFO could be completely eluted from silica gel, up to concentrations of 25–50 μM in the sample, as assessed by HPLC analysis of several fractions of the eluate.

For the HPLC detection of DFO it was necessary to convert it into FO by the addition of a suitable amount of $FeSO_4$. In fact DFO, at μM concentrations, is converted to FO during the HPLC analysis, presumably through a reaction with iron ions liberated from the stainless steel plumbing system of the HPLC apparatus by the acetic acid component of the solvent system [24]. At higher concentrations, however, DFO can be detected by HPLC as a peak well separated from that of FO.

Ferrioxamine present in the sample was eluted from the silica gel column with 10 ml of a second solvent mixture made up by methanol:acetonitrile:H_2O:glacial acetic acid:33% ammonia (49:49:2:0.35:0.15, by vol.). Under these conditions the elution of FO was complete, as assessed by HPLC analysis of several fractions of the eluate. The eluate was dried under nitrogen, the residue was dissolved in a small volume of the solvent used as the mobile phase (see below) and analyzed by HPLC.

HPLC was performed with a Du Pont Liquid Chromatograph with a variable wavelength UV detector. Operating conditions used: silica gel column (Lichrosorb Si 60, 5μm, Merck; 250 × 4 mm); mobile phase, methanol:acetonitrile:H_2O:glacial acetic acid:10 N NaOH (45:45:10:0.2:0.1, by vol.); flow rate, 1.5 ml/min; UV photometer at 229 nm.

A standard solution of DFO in iron-free KCl–Tris–maleate buffer and standard solutions of $FeSO_4$ in H_2O were prepared each experimental day. They were mixed in such a way to have final solutions containing 25 μM DFO and 1–10 μM $FeSO_4$. The standard samples were processed by silicic acid column chromatography and HPLC as was done for the experimental samples. A plot of peak areas against iron concentration in the standard samples is reported in Fig. 1. Blank samples were processed as standard samples.

RESULTS

As shown in previous reports from our and other laboratories [15–17], the administration of a toxic dose of allyl alcohol to mice results in a very rapid depletion of hepatic GSH, which is maximal within 15–20 min [16, 17] and which is followed by the development of lipid peroxidation and liver necrosis. In the intoxicated animals in which the parameters of liver injury were followed the hepatic GSH level was 0.9 ± 0.2 μmol/g liver (vs 5.4 ± 0.2 in controls) at 1–2 hr after the intoxication; the hepatic MDA content (a measure of lipid peroxidation, Ref. 25) was 43.8 ± 6.9 nmol/g liver (vs virtually 0 in the controls); and the serum glutamate–pyruvate transaminase level was 894 ± 200 U/l (vs 51 ± 26 in the controls).

As previously mentioned, a severe hemolysis was observed in about 50% of the allyl alcohol-intoxicated animals. As shown in Table 1, a marked decrease in erythrocyte GSH was seen in all the intoxicated animals. Such a decrease was higher in the animals showing substantial hemolysis (Group 2) compared with the animals in which hemolysis

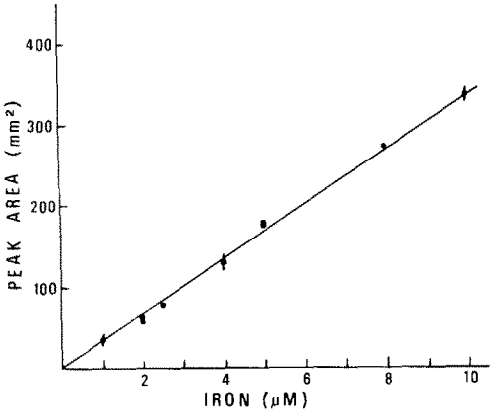

Fig. 1. Plot of HPLC peak areas of FO obtained by mixing standard solution of DFO and $FeSO_4$ against the indicated concentrations of $FeSO_4$ in "iron-free" KCl–Tris–maleate buffer pH 7.4. The final solutions contained DFO at the concentrations of 25 μM. The standard samples were processed by silicic acid column chromatography and HPLC as described in Materials and Methods section. The recovery of FO after the chromatographic process was complete as assessed by the comparison of HPLC peak areas shown in the figure with those obtained after mixing equimolar amount of DFO and $FeSO_4$. Using $FeCl_3$ instead of $FeSO_4$, we obtained substantially the same results.

10 mM or more, did not induce either GSH depletion, lipid peroxidation or hemolysis, acrolein reproduced the effects seen in the *in vivo* studies. In fact acrolein added to mouse erythrocytes at the concentration of 6 mM, induced a dramatic GSH depletion (Fig. 2, panel A), which was followed by the development of lipid peroxidation (Fig. 2, panel B). The lysis of erythrocytes started approximately 30 min after the appearance of MDA (Fig. 2, panel C). The addition of antioxidants such as Trolox C (a lower homolog of vitamin E) or DFO (an iron chelator) completely prevents both lipid peroxidation and hemolysis (Fig. 2, panels B and C). Trolox C and DFO had no effect on GSH content of erythrocytes (Fig. 2, panel A). Thus, it is clear from the data of Fig. 2 that acrolein, acting *in vitro* on erythrocytes, dramatically lowers erythrocyte GSH content. This is followed within 15–30 min by initiation of lipid peroxidation, and, after another 30–45 min, hemolysis is evident. If lipid peroxidation is prevented by an antioxidant or by iron chelation, hemolysis does not occur despite dramatically depressed GSH levels. Acrolein at the concentration of 3 mM was ineffective in inducing lipid peroxidation and hemolysis; the per cent decrease in erythrocyte GSH was 81.4 ± 0.7 at the various incubation times, whereas when, in simultaneous experiments, 6 mM acrolein was used, the per cent GSH decrease was 91.5 ± 0.7.

Table 1. Table 1. Hemolysis, erythrocyte GSH, and plasma malonic dialdehyde (MDA) level in allyl alcohol-treated mice

	No. of animals	Hemolysis (% relative to 100% hemolysis)	GSH (nmol/ml)	MDA (nmol/ml)
Controls	10	0.6 ± 0.07	831.7 ± 32.6	—
Allyl alcohol (Group 1)	19	0.7 ± 0.06	271.9 ± 16.7	3.5 ± 0.9
Allyl alcohol (Group 2)	21	19.8 ± 3.4	172.5 ± 13.1*	27.1 ± 3.3

Allyl alcohol was given i.p. at the dose of 1.5 mmol/kg body wt. The animals were sacrificed 1–2 hr after the intoxication. Results are given as means ± SE.

With respect to hemolysis, Group 1 is defined as having a level of hemolysis ≤ 1% of complete hemolysis. Group 2 is defined as having a level of hemolysis ≥ 1% of complete hemolysis.

Control values for MDA were subtracted from those of the intoxicated animals.

* The erythrocyte GSH was measured in 10 animals only. The value is significantly different from the corresponding value of Group 1 ($P < 0.001$).

was virtually absent (≤ 1%) (Group 1). Likewise, MDA was detected in the plasma of almost all the intoxicated animals. However, the MDA level was much higher in the animals showing hemolysis (Group 2). In the latter animals, a significant decrease in arachidonic and docosahexaenoic acids was found in erythrocyte phospholipids (Table 2). The appearance of MDA in plasma as well as the decrease in the most highly unsaturated fatty acids in erythrocyte phospholipids are indicative of the development of lipid peroxidation in erythrocyte membrane.

In an attempt to investigate the mechanism by which allyl alcohol induces GSH depletion and lipid peroxidation in erythrocytes, we studied the effects of allyl alcohol and its major metabolites on red blood cells *in vitro*. It was found that while allyl alcohol and acrylic acid, even at concentrations of

It has been suggested that the cellular damage induced by oxidative stress is mediated by an "iron delocalization", that is, the release of free iron from iron stores [26, 27]. Such iron may promote pathological processes, such as lipid peroxidation [26–28]. Therefore, we measured the amount of free iron (DFO-chelatable) in the *in vitro* system in which acrolein interacts with erythrocytes. As can be seen, the amount of free iron progressively increases during the incubation (Table 3; B), as documented by the increase in the DFO–iron complex measured by HPLC. Lipid peroxidation did not occur in these samples because of the addition of DFO (at 15 min of incubation) necessary to chelate free iron. When DFO was added at the end of the incubation, that is, after the occurrence of lipid peroxidation (Table 3; C), the amount of iron detectable as a DFO–iron complex was somewhat higher than in the samples

Table 2. Fatty acid composition of erythrocyte phospholipids in allyl alcohol-treated mice which showed significant hemolysis

	Fatty acids						
	16:0	16:1	18:0	18:1	18:2	20:4	22:6
Controls (3)	29.9 ± 1.4	0.7 ± 0.1	14.3 ± 0.3	13.6 ± 0.6	13.5 ± 0.7	20.2 ± 0.5	7.9 ± 0.4
Allyl alcohol (3)	36.1 ± 0.7	1.4 ± 0.2	15.7 ± 1.0	14.6 ± 0.6	12.1 ± 0.9	15.0 ± 0.6*	5.2 ± 0.2*

Allyl alcohol was given i.p. at the dose of 1.5 mmol/kg body wt. In allyl alcohol-treated mice the hemolysis was 49.4 ± 8.3% of complete hemolysis and the MDA level in plasma was 37.9 ± 3.7 nmol/ml. Results are given as means ± SE. The number of animals is reported in parentheses.

* Significantly different from controls: $P < 0.001$.

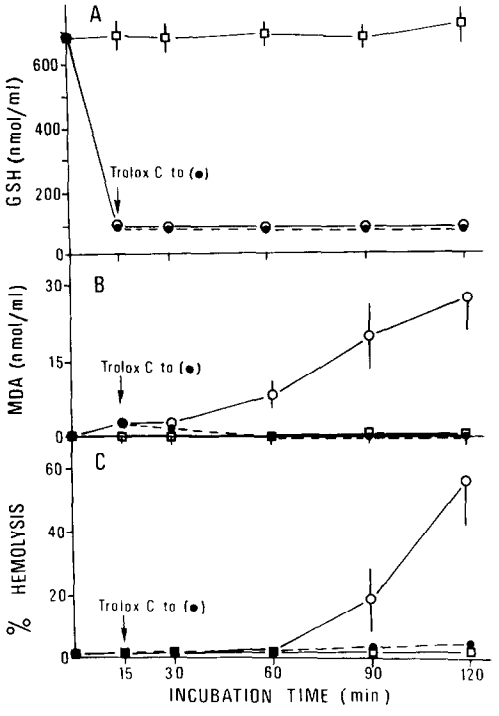

Fig. 2. GSH (panel A), MDA formation (panel B) and hemolysis (panel C) in control erythrocytes (□—□) or in erythrocytes incubated with acrolein (6 mM) (○—○). Dashed lines (●- - -●) indicate the values obtained when Trolox C (50 μM) was added to the incubates at 15 min of incubation. The values obtained with DFO (50 μM) addition were virtually identical to those for Trolox C. Results represent the means ± SE of 4 experiments. Where not reported, standard error bars were narrower than the symbols used.

(Table 3; B) in which lipid peroxidation did not occur. It seems, therefore, that acrolein induces a release of iron from iron complexes in erythrocytes. Such free iron appears to promote lipid peroxidation, which, in turn, leads to a further release of chelatable iron.

DISCUSSION

The present work demonstrates that allyl alcohol intoxication produces, in about half of the animals, a severe hemolysis. The pathogenetic mechanism of such hemolysis seems to have some similarity with that of the liver cell death induced by allyl alcohol itself [16, 17] and probably other GSH-depleting agents [18]. In fact, a rapid fall in GSH also occurs in erythrocytes, as well as in the hepatocytes, after allyl alcohol administration; hemolysis develops in the animals in which the erythrocyte GSH depletion is more marked. The possibility that hemolysis is produced by lipid peroxidation in erythrocyte membrane is supported by the appearance of MDA in plasma as well as by the decrease in arachidonic and docosahexaenoic acids in erythrocyte phospholipids. Thus in the erythrocyte, as in the liver cell, GSH depletion (and probably other events connected with the metabolism of the toxin) is followed by the development of lipid peroxidation and cell destruction.

What is difficult to explain is how allyl alcohol induces GSH depletion and lipid peroxidation in erythrocytes. In fact, allyl alcohol is metabolized in the liver or possibly in other tissues provided with alcohol dehydrogenase. No alcohol dehydrogenase has been shown in mammalian erythrocytes [29]. Furthermore, in the *in vitro* studies of the present report, allyl alcohol was found to be completely ineffective when added to erythrocyte suspensions. The main allyl alcohol metabolite, acrolein, on the other hand, reproduced the effects of allyl alcohol intoxication in incubated erythrocytes. It is, however, difficult to imagine that free acrolein is released from the liver into the blood compartment in sufficient amounts to interact efficiently with erythrocytes. Possibly the diffusing metabolites are transported in the form of conjugates to erythrocytes in which the adducts are converted to reactive metabolites.

The *in vitro* experiments show that acrolein induces a very rapid GSH depletion in erythrocytes. This is followed by the appearance of lipid peroxidation and, after an additional 30 min of incubation, by the development of hemolysis. Prevention of lipid peroxidation by an antioxidant (Trolox C) or an iron chelator (desferrioxamine) also prevents hemolysis even if the GSH level is dramatically depressed. It seems, therefore, that the acrolein-induced hemolysis is mediated by lipid peroxidation.

A great deal of experimental evidence [26, 27, 30, 31] suggests that in a number of pathological conditions in which oxidative stress is

Table 3. Release of free iron and lipid peroxidation (malonic dialdehyde (MDA) formation) in erythrocytes incubated with acrolein

	Incubation time (min)	Free iron (nmol/ml)	MDA formation (nmol/ml)	Hemolysis (%)
A. 1. Erythrocytes (no acrolein)	0	2.6 ± 0.3	—	1.6 ± 0.1
2. Erythrocytes (no acrolein), DFO (added after 15 min of incubation)	120	2.6 ± 0.2	0	2.1 ± 0.4
B. 1. Erythrocytes + acrolein	0	2.7 ± 0.3	—	1.9 ± 0.2
2. Erythrocytes + acrolein + DFO (added after 15 min of incubation)	30	3.3 ± 0.2	0	1.9 ± 0.3
3. Erythrocytes + acrolein + DFO (added after 15 min of incubation)	60	3.5 ± 0.2	0	2.5 ± 0.1
4. Erythrocytes + acrolein + DFO (added after 15 min of incubation)	120	5.5 ± 0.2	0	2.9 ± 0.2
C. 1. Erythrocytes + acrolein (no DFO present during incubation)	0	2.7 ± 0.3	—	1.7 ± 0.2
2. Erythrocytes + acrolein (no DFO present during incubation)	60	5.3 ± 0.2	16.1 ± 4.4	13.4 ± 4.4
3. Erythrocytes + acrolein (no DFO present during incubation)	120	9.5 ± 1.0	37.8 ± 4.2	95.6 ± 2.9

The release of free iron was measured as desferrioxamine (DFO)-chelatable iron. Desferrioxamine was added after 15 min of incubation in the samples A 2 and B 2, 3 and 4. Desferrioxamine was added at 0 time in the samples A 1 and B 1. Desferrioxamine was added at 0, 60 and 120 min of incubation in the samples C 1, 2 and 3, respectively. Acrolein was added in the samples B and C at the concentration of 6 mM. Results represent the means ± SE of 5 experiments.

Zero time values for MDA were subtracted from those of the incubated samples.

involved, iron is released from iron stores and induces cellular damage both by generating hydroxyl radicals through the Fenton reaction and by promoting lipid peroxidation. Normally iron is transported and stored in specific proteins (ferritin, transferrin, lactoferrin and haem proteins) which prevent or minimize its reaction with reduced oxygen metabolites [32]. However, in spite of these safeguards, increasing evidence suggests that reactive iron becomes available during disease states. Studies have shown [33–36] that the specific iron chelator DFO can modify tissue changes observed in inflammatory, degenerative and ischemic damage. In model systems it has been shown [37] that the superoxide anion (O_2^-) generated by xanthine oxidase promotes release of iron from added ferritin and that such released iron can promote lipid peroxidation in phospholipid liposomes. Also, it has been shown [38] that liver microsomes contain ferritin and that O_2^- generated by paraquat via redox cycling releases iron from added ferritin. Such iron would then serve to promote lipid peroxidation.

An interesting feature of the present study is the demonstration that iron is released in a "free" (desferal chelatable) form during the incubation of erythrocytes with acrolein. To our knowledge, this is the first direct demonstration of an iron release during interactions of cells with GSH-depleting agents. An obvious question is where the released iron comes from. A possibility is that iron is released from haemoglobin during conditions in which an oxidative stress is imposed on red blood cells. It has been shown [39, 40] that H_2O_2 and organic peroxides release iron from haemoglobin. Such free iron is then available for induction of lipid peroxidation and production of hydroxyl radicals by the Fenton reaction.

As shown in Table 3 (B; 2–4) the release of iron can occur in the absence of lipid peroxidation, when the latter is inhibited by the presence of DFO in the system. It is therefore possible that the release of iron is the cause of the induction of lipid peroxidation. However, when lipid peroxidation is allowed to develop in the absence of DFO during the incubation (Table 3: C; 2,3), the iron release is higher than in the case in which lipid peroxidation is inhibited by DFO (Table 3: B; 2,3,4). Thus, lipid peroxidation leads to a further release of iron. In liver microsomes incubated in the NADPH–Fe-dependent system it has been shown [41–43] that the development of lipid peroxidation leads to a rapid degradation of heme and hemoprotein cytochrome P-450. If some heme degradation occurs as a consequence of lipid peroxidation in erythrocytes, then some iron may be released from degraded heme and may amplify the extent of lipid peroxidation.

It must be recognized, however, that from the present results it is not sufficiently clear whether, during the interaction of erythrocytes with acrolein, iron is released in sufficient amount to induce lipid peroxidation or whether the released iron just amplifies the latter. Further studies are needed to clarify this point. However, the observation that iron is released in cells interacting with a GSH-depleting agent may be of interest in the explanation of the pathogenetic mechanisms of cell damage induced by oxidative stress.

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