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SHORT COMMUNICATIONS

Loss of membrane protein thiols and lipid peroxidation in allyl alcohol hepatotoxicity

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Allyl alcohol is metabolized by cytosolic alcohol dehydrogenase to acrolein, an unsaturated aldehyde belonging to the class of 2-alkenals provided of high reactivity towards sulphydryl groups and other nucleophiles [1]. Cellular glutathione is in fact primarily involved in the reaction, which results in a dramatic depletion of glutathione stores [2, 3], and it has been suggested that the alkylation of nucleophilic groups of cellular macromolecules effected by acrolein after glutathione depletion is the event actually leading to cell injury [4]. However, recent reports have suggested that a major role in the production of allyl alcohol-induced hepatotoxicity might be played by lipid peroxidation [5–10], as in the case of other glutathione-depleting agents such as bromobenzene, acetaminophen and diethylmaleate (see Ref. 11 for review). During the peroxidation of membrane phospholipids, 4-hydroxynonenal and other reactive aldehydes provided with highly cytopathological activities are produced (see Ref. 12 for review). We have indeed shown the occurrence of 4-hydroxynonenal and other lipid-derived cytotoxic carbonyls *in vivo*, in the liver of animals intoxicated with bromobenzene [13] and allyl alcohol itself [9]. Hydroxyalkenals exert their cytopathological activities through interactions with cellular nucleophiles [1, 12], among which protein thiol groups represent a major target. In addition, during lipid peroxidation proteins are exposed to a wide range of lipid free radical species, which are capable of oxidizing protein sulphydryls, thus promoting the formation of disulphide bridges, and even inducing protein fragmentation [14].

The loss of protein sulphydryls has been implicated in the loss of cell viability in several experimental conditions (see Ref. 15 for review), and attempts have been made to identify distinct pools of protein thiols whose integrity is essential for the maintenance of cell viability. In this perspective, allyl alcohol intoxication—with its dual toxic aggression to cells (alkylation of macromolecules, lipid peroxidation)—represents a particularly interesting model for studying both the role played by lipid peroxidation and the involvement of different cell protein thiol compartments in the production of cell injury. In fact, lipid peroxidation-derived aldehydes originate and accumulate in the lipid domain of membranes [12, 16, 17]; the possibility therefore exists that lipid peroxidation—through the formation of reactive aldehydes or other lipid radical species—may cause a selective loss of protein thiols in cellular membranes, and that this event—rather than protein thiol alkylation by cytosol-borne acrolein—may in turn relate with liver injury. This possibility was verified in the present study.

Materials and Methods

'In vivo' *experiments*. Male NMRI albino mice (Charles River, Italy), weighing 25–30 g and maintained on a pellet diet, were starved overnight before receiving allyl alcohol (1.5 mmol/kg body wt, i.p.) diluted in saline, or an equivalent volume of saline. The animals were killed by exsanguination 1.5 hr after the intoxication. In some experiments, desferrioxamine (Desferal®, kindly supplied by Ciba Geigy, Switzerland), dissolved in saline, was administered to mice at the dose of 250 mg/kg body wt, i.p., 20 min after intoxication with allyl alcohol. Liver injury was assessed by measuring the levels of serum glutamate-pyruvate transaminase activity (optimized UV-enzymatic method, Sclavo, Italy). Lipid peroxidation was assessed by measuring the hepatic content of malonaldehyde (MDA*), after direct reaction of acid tissue extracts with thiobarbituric acid at 100° for 10 min, as described previously [18]. In fact, we have previously shown that the direct assay of tissue MDA content strictly correlates with other more sophisticated procedures for detection of lipid peroxidation *in vivo* [18]. Hepatic glutathione was determined as acid-soluble SH groups, according to Sedlak and Lindsay [19]. Protein was determined according to Lowry *et al.* [20].

Protein thiols were determined separately in two liver fractions, one corresponding to cytosol (105,000 *g* supernatant), the other including cell membranous structures altogether (105,000 *g* pellet, washed), obtained from 20% (w/v) liver homogenates in 0.25 M sucrose, 6 mM EDTA (pH 7.4). For the determination of protein thiols, proteins were precipitated with 5% trichloracetic acid, resuspended in a 0.2 M Tris, 20 mM EDTA buffer, pH 8.2 [19] containing 1% sodium dodecylsulfate, and reacted with 0.2 mM (final concn) 5,5-dithiobis-2-nitrobenzoic acid. After 20 min, absorbance of samples at 412 nm was measured against internal blanks. Data were expressed as nmol-SH/mg protein, calculated on the basis of a molar extinction coefficient of 13,100 [19].

'In vitro' *experiments*. Mitochondria and microsomes were isolated from 20% (w/v) liver homogenates in 50 mM Tris–maleate, 0.15 M KCl, 3 mM EDTA buffer, pH 7.4, as reported [21]. Isolated fractions were resuspended in the same buffer to have the equivalent of 0.2 g liver/mL and incubated for 30 min at 37° with different concentrations of acrolein (Fluka, Switzerland). In parallel experiments, unfractioned liver homogenates were dialysed by a Visking dialysis tube (Serva Feinbiochemica, F.R.G.) for 24 hr at 4° against the basic buffer, in order to remove non-protein soluble thiols (mainly glutathione). Liver homogenates were then incubated with increasing concentrations of acrolein. Cell subfractioning was performed in these experiments after the incubation. Mitochondria, microsomes and (dialysed) cell soluble fraction were obtained, and the protein thiol content of each fraction was determined.

Results

In livers from allyl alcohol-intoxicated mice, protein thiols were studied in the cell soluble fraction as compared to the cell membrane fraction, and an experimental condition was investigated in which lipid peroxidation following allyl alcohol was prevented. This was done by treating some of the intoxicated animals with desferrioxamine 20 min after allyl alcohol administration, i.e. at a time when the glutathione depletion resulting from the metabolism of the toxin has completed [9]. Indeed, in both groups of animals (allyl alcohol- and allyl alcohol/desferrioxamine-treated) a

* Abbreviations: EDTA, ethylenediaminetetracetic acid; Tris, tris(hydroxymethyl)aminomethane; SGPT, serum glutamate-pyruvate transaminase.

marked glutathione depletion (more than 90%) was observed (Fig. 1A), indicating that the desferrioxamine treatment did not likely modify allyl alcohol metabolism. As expected on the basis of previous reports, allyl alcohol caused the appearance of lipid peroxidation (Fig. 1B), which was associated with liver damage (Fig. 1C). Desferrioxamine suppressed lipid peroxidation (Fig. 1B), and largely prevented the rise of SGPT values (Fig. 1C).

In mice presenting with allyl alcohol-induced lipid peroxidation, a significant protein thiol loss was observed both in the cell soluble fraction (approx. −30%, Fig. 1D) and in membranes (approx. −30%, Fig. 1E). Investigation of the distribution of the membrane protein thiol loss in three major cell membrane fractions showed that microsomal and mitochondrial membranes were primarily involved, while nuclear membranes were affected at a considerably lower extent (Fig. 2). When allyl alcohol-induced lipid peroxidation was prevented by desferrioxamine treatment (Fig. 1B), the extent of protein thiol decrease in membranes was markedly reduced (approx. −7%, Fig. 1E), whilst a significant thiol loss was still present in proteins of the cell soluble fraction (approx. −21%, Fig. 1D).

Thus, assuming that the loss of soluble protein thiols observed in the absence of lipid peroxidation (desferrioxamine-treated animals) is essentially due to alkylation by acrolein produced during allyl alcohol metabolism, acrolein alone does not seem able to affect protein thiols of membranes significantly, at least in our experimental conditions. An attempt to clarify this point was made *in vitro*, by exposing different preparations of cell subfractions to increasing concentrations of acrolein. With isolated mitochondria and microsomes, a linear decrease of membrane protein thiols was actually observed (Fig. 3A). The possibility that membrane protein thiols are in some way less reactive towards acrolein is therefore unlikely. On the other hand, different results were obtained by treating dialysed (glutathione-depleted) liver homogenate with acrolein, a situation in which cell membranes were challenged with acrolein in the presence of cytosolic, soluble proteins; three cell subfractions (mitochondria, microsomes and soluble fraction) were obtained in this case at the end of the incubation. Under these conditions, acrolein (up to high concentrations, 25 mM) caused only minor protein thiol losses both in mitochondria and in microsomes, while protein thiols of the soluble fraction were remarkably affected (Fig. 3B).

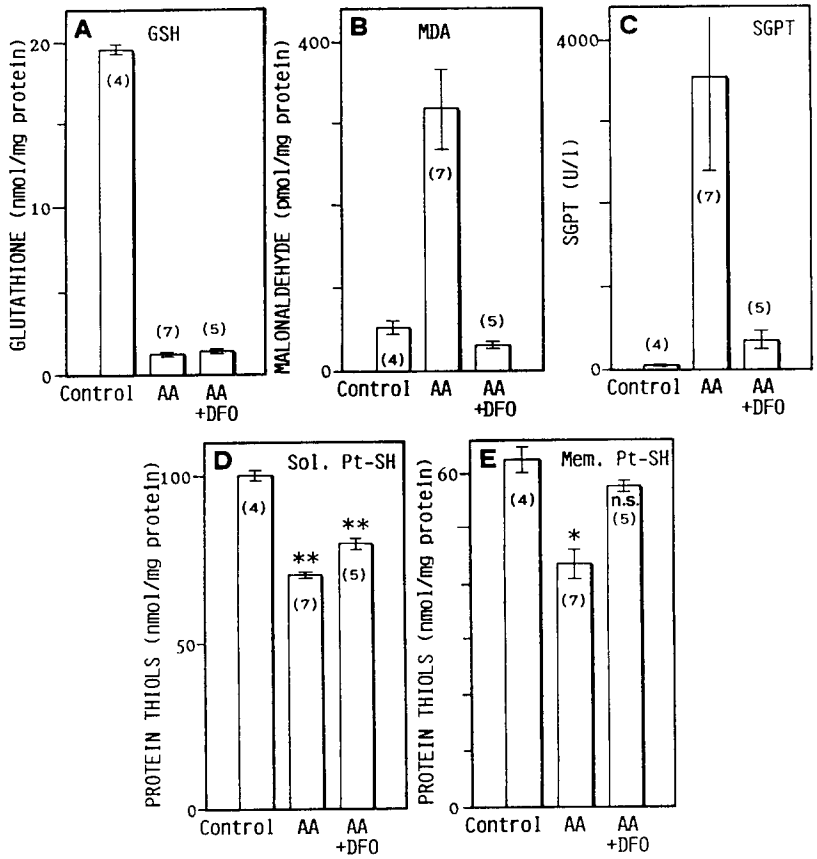

Fig. 1. Hepatic glutathione (GSH, A), lipid peroxidation (hepatic MDA contents, B) liver injury (SGPT values, C) and protein thiols in soluble (D) as compared to membranous (E) fraction of mouse liver homogenates, in controls, allyl alcohol-intoxicated (AA) and allyl alcohol-intoxicated-desferrioxamine-treated (AA + DFO) mice. All animals were killed 90 min after the intoxication. Allyl alcohol diluted in saline was given intraperitoneally (1.5 mmol/kg). Desferrioxamine was given in a single administration of 250 mg/kg, in saline, i.p., 20 min after allyl alcohol intoxication. Data reported represent means ± SE. The number of animals is reported in parentheses. *$P < 0.01$, **$P < 0.001$, with respect to control; n.s., not significantly different from control.

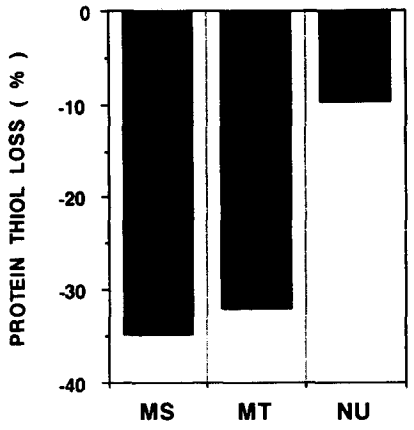

Fig. 2. Distribution of membrane protein thiol loss among three membrane fractions 90 min after allyl alcohol intoxication (MS, microsomes, MT, mitochondria, NU, nuclei). Membrane fractions were obtained as reported [18]. Thiol loss is expressed as per cent loss in each fraction with respect to control; determinations were performed in two pools (control, allyl alcohol), including the livers of three animals each.

Discussion

Hepatoxicity of allyl alcohol has been shown to depend on its metabolism by cytosolic alcohol dehydrogenase to acrolein [22]. It has been envisaged that acrolein produced intracellularly reacts preferentially with glutathione, and then—once glutathione is depleted—with protein thiols and other nucleophilic groups of cellular macromolecules, thus leading to cell injury [4]. However, data reported in the present study do not support the hypothesis that alkylation of macromolecules by acrolein is a major factor in the production of acute allyl alcohol hepatoxicity, since the loss of protein thiols observed—in absence of lipid peroxidation—in the soluble, cytosolic fraction was not associated with production of liver injury. The latter was in fact associated with the development of lipid peroxidation and was protected by desferrioxamine-treatment, which did not significantly reduce the extent of thiol loss in soluble proteins with respect to controls. On the contrary, prevention of lipid peroxidation by desferrioxamine resulted in preservation of protein thiol levels in membranes and protection of cellular injury. It seems therefore that, although highly reactive with cytosolic soluble proteins, acrolein produced intracellularly is unable to significantly affect protein thiols of membranes, even at a time when cellular glutathione has been almost completely depleted. This aspect was investigated with isolated liver subfractions.

The *in vitro* experiments reported in Fig. 3 actually show that indeed acrolein is able to react with protein thiols of isolated membranes, but this interaction is largely prevented in the presence of soluble, cytosolic proteins, when a preferential interaction of acrolein with soluble proteins likely occurs. This effect could at least partly explain the observed preservation of membrane protein thiols *in vivo* during active metabolism of allyl alcohol to acrolein, in absence of lipid peroxidation. On the other hand, many of the reactive compounds generated during the peroxidative breakdown of membrane phospholipids—due to their high lipophilicity—are essentially confined within the lipid domain of the membrane itself, where their local concentration can rapidly reach levels high enough to attack the hydrophobic regions of membrane enzymes [16, 17]. Indeed, loss of protein thiols depending on the development of lipid peroxidation or on the exogenous addition of preformed lipid peroxidation products has been repeatedly demonstrated [23–26].

The maintenance of protein thiols has been reported to be critical for protection against viability loss from allyl alcohol [27]. Protein thiol alterations have been shown to result in perturbation of cell Ca^{2+} homeostatis. Loss of essential membrane protein thiols in the impairment of Ca^{2+} transport across cellular membranes and loss of cell viability (see Ref. 28 for review). On the other hand, it is well documented that lipid peroxidation can impair cell Ca^{2+} homeostasis. Ca^{2+}-sequestering activity of liver microsomes has been shown to be inhibited by lipid peroxidation products such as 4-hydroxyalkenals, and the extent of such inhibition was correlated with the amounts of 4-hydroxyalkenals bound to microsomal protein [29]. Interestingly, hepatotoxicity of the alkaloid senecionine has been recently shown to depend on the formation of a hydroxyalkenal metabolite which inhibits Ca^{2+} sequestration through interaction with membrane protein thiols [30]. In addition, we have previously reported that bromobenzene intoxication in mice induces lipid peroxidation-

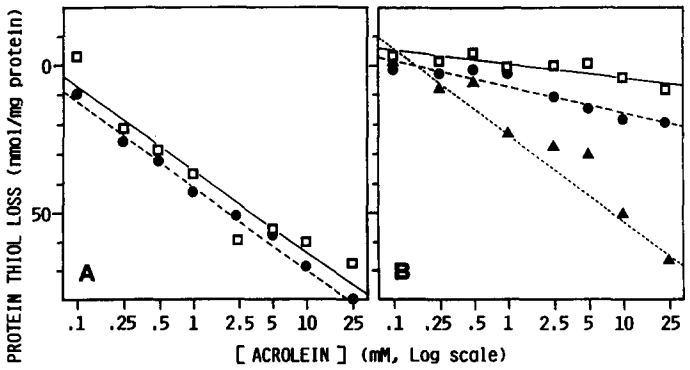

Fig. 3. (A) Loss of protein thiols in isolated mitochondria (□) and microsomes (●), each separately incubated with increasing concentrations of acrolein. (B) Loss of protein thiols in mitochondria (□), microsomes (●) and post-microsomal supernatant fraction (▲), isolated after the incubation of whole, dialysed liver homogenate with increasing concentrations of acrolein. Incubations were carried out at 37° for 30 min. Cell subfractionation was performed as reported in Materials and Methods, from liver homogenates including 10–11 mouse livers each. In the case of panel B, liver homogenate was dialysed (see Materials and Methods) in order to remove cellular glutathione prior to the incubation with acrolein, and it was ascertained that dialysis had removed more than 90% of reduced glutathione.

dependent membrane protein thiol loss, depression of intracellular Ca^{2+}-sequestration and hepatocellular injury [24].

In summary, the data reported suggest that—following initiation of lipid peroxidation—membrane protein thiols can be attacked by lipid-derived radicals and/or reactive, lipid-soluble aldehydes like 4-hydroxynonenal and other hydroxyalkenals originated within the lipid core of cell membranes, resulting in a membrane protein thiol loss which is in turn associated with the development of hepatocellular injury.

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Effects of polychlorinated biphenyls on cholesterol and ascorbic acid metabolism in primary cultured rat hepatocytes

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We previously reported that dietary polychlorinated biphenyls (PCBs*) produced hypercholesterolemia, and increased urinary and tissue levels of ascorbic acid accompanied the induction of drug metabolizing enzymes in rats [1]. A diet of PCBs or other xenobiotics served to stimulate incorporation of ^3H_2O into liver cholesterol together with an increase in the activity of liver HMG-CoA reductase (EC 1.1.1.34), suggesting that cholesterol synthesis was encouraged [2, 3]. Horio and Yoshida [4] reported that UDP-glucuronyl transferase (EC 2.4.1.17) activity was increased by PCBs or other xenobiotic feeding accompanied by a marked increase in urinary and liver ascorbic acid levels in rats. The precise mechanisms by which PCBs induced these changes related to cholesterol and ascorbic acid metabolism is not yet well understood.

Recently, primary cultured hepatocytes have been widely used in metabolic studies [5, 6]. One of the advantages of this model is to allow the study of metabolic expression of liver cells in a culture medium of defined composition, independently of hormonal or nutritional variations. To our knowledge, no one has reported the effects of PCBs on cholesterol and ascorbic acid metabolism in primary cultured hepatocytes. Thus, the present study was designed to investigate the influence of PCBs on cholesterol and ascorbic acid metabolism by the rat liver parenchymal cells in primary culture.

Materials and Methods

Parenchymal hepatocytes were isolated from adult male Wistar rats, weighing about 200 g, by perfusion of the liver *in situ* with collagenase, essentially as described by Seglen [7]. The viability of cells after isolation as measured by the ability of the cells to exclude trypan blue was usually greater than 90%. The culture medium was Williams medium E supplemented with 10% fetal bovine serum, 10^{-9} M insulin, 10^{-8} M dexamethasone, penicillin 100 units/mL, streptomycin 100 μg/mL and fungizone 0.25 μg/mL. Inocula of 1×10^5 cells/0.2 mL/cm^2 were seeded into collagen-coated petri dishes (Corning, 10-mm plate), and cultured as monolayers in a humidified incubator at 37° under 5% CO_2 in air. The medium was changed after the first 4 hr, and then 6 μM PCB (Aroclor 1248, Mitsubishi Monsanto, Tokyo) was added to the medium after 24 hr in culture, and cells were harvested with a rubber policeman at 48 hr. The composition of Aroclor 1248 [8] is 2% dichlorobiphenyl, 18% trichlorobiphenyl, 40% tetrachlorobiphenyl, 36% pentachlorobiphenyl and 4% hexachlorobiphenyl. The dosage (6 μM) of PCBs was based on previous pharmacological studies [9, 10] and preliminary experiments. PCBs were first dissolved in small amounts of dimethylsulfoxide, and this solution was then added to the growth medium as described previously [10]. Cell number was analysed by the method of Nakamura *et al.* [11]. Cells were incubated in Hanks solution containing 5 mM NH_4Cl and the amount of urea formed was measured by the method of Geyer and Dabich [12]. A sample of 5×10^6 cells was homogenized in 0.4 mL of 5 mM phosphate buffer (pH 7.8) containing 5 mM 2-mercaptoethanol and centrifuged, and the supernatant was used for the assay of tyrosine transaminase (EC 2.6.1.5) activity as described previously [13]. Protein was determined by the method of Lowry *et al.* [14]. Cellular ascorbic acid was measured by 2,4-dinitrophenylhydrazine method [15]. Cellular cholesterol content was measured by an enzymatic colorimetric method (Monotest Cholesterol, CHOD-PAP-method, Boehringer Mannheim GmbH, Mannheim, F.R.G.). HMG-CoA reductase activity was measured according to the method of Ide *et al.* [16]. Cell monolayers were washed twice with cold Dulbecco's phosphate-buffered saline before they were gently scraped from the dishes and centrifuged at 1000 *g* collected in homogenizing tubes in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.25 M sucrose, 0.075 M nicotinamide, 2.5 mM EDTA and 1 mM dithiothreitol. Cells were homogenized with Polytron. Cell homogenates were centrifuged at 10,000 *g* for 20 min. The supernatants were then recentrifuged at 105,000 *g* for 60 min. Microsomal pellets were used for the assay of HMG-CoA reductase activity. Aryl hydrocarbon hydroxylase (EC 1.14.14.2) activity was measured according to the method of Murakami *et al.* [9] and Nebert and Gelboin [17]. Cell monolayers were washed twice with cold 0.05 M Tris–HCl (pH 7.4) containing 0.15 M NaCl before they were gently scraped from the dishes and centrifuged at 1000 *g* collected in homogenizing tubes in 0.05 M Tris–HCl (pH 7.4) containing 0.25 M sucrose. Cells were homogenized with a Polytron. Microsomal pellets were used for the assay of aryl hydrocarbon hydroxylase activity. UDP-glucuronyl transferase activity was analysed according to Henderson [18]. The cell pellets were collected in cold Dulbecco's phosphate-buffered saline and homogenization was carried out in 0.25 M sucrose/0.05 M Tris–HCl (pH 7.5) with a Polytron. The methods for the preparation of microsomal pellets used were the same as those for aryl hydrocarbon hydroxylase. Microsomal pellets were used for the assay of UDP-glucuronyl transferase activity. For the incorporation of [1−^{14}C]acetate into hepatocyte cholesterol, freshly isolated hepatocytes were cultured for 24 hr under standard conditions, and then PCB (6 μM) was added to the medium. After 24 hr incubation, the cells were incubated with 6 mL

* Abbreviations: PCB, polychlorinated biphenyls; HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A.