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Allyl Alcohol Liver Injury: Suppression by Ethanol and Relation to Transient Glutathione Depletion

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Abstract: Rats metabolized a sublethal gastric dose (0.73 mmol/kg) of allyl alcohol (AlOH) within 10–15 min. Oxidation of AlOH to acrolein was accompanied by an equally rapid, but only transient depletion of hepatic reduced glutathione (GSH). GSH was restored to levels above normal within 5 hrs. Simultaneously, AlOH provoked marked elevation of alanine aminotransferase, γ-glutamyl transpeptidase, and glutamate dehydrogenase activities in plasma and formation of lesions mainly in the periportal regions of the liver. Inhibition of alcohol dehydrogenase by 4-methyl pyrazole completely counteracted these effects. On the other hand, attempts to potentiate the toxicity of acrolein by the aldehyde dehydrogenase inhibitor cyanamide enhanced only the release of alanine aminotransferase. Co-administration of ethanol (3 g/kg) inhibited the rate of AlOH oxidation by more than 90%. Although with ethanol GSH remained depleted for several hours, the release of enzymes was markedly suppressed and the histologic changes completely prevented. These results indicate that the rapid rate of acrolein formation, rather than persistently lowered GSH content, is crucial in the hepatotoxicity of AlOH. They also suggest, that oxidation of acrolein via aldehyde dehydrogenase does not represent a major pathway for its detoxication *in vivo*.

As opposite to most other hepatotoxins, allyl alcohol (AlOH, 2-propen-1-ol) causes hepatic injury primarily in the periportal region of the liver lobulus (Rees & Tarlow 1967). The toxicity requires oxidation of AlOH by alcohol dehydrogenase to highly reactive acrolein (Serafini-Cessi 1972), that binds covalently to, and thereby inactivates, essential cellular macromolecules (Reid 1972).

The periportal toxicity has been ascribed to higher alcohol dehydrogenase activity in periportal than in perivenous hepatocytes (Rees & Tarlow 1967), but studies showing even sublobular distribution or perivenous dominance of alcohol dehydrogenase activity (Morrison & Brock 1967; Bengtsson *et al.* 1981; Väänänen *et al.* 1984) indicate that some other factors are involved.

To investigate alternative mechanisms we have initiated studies on the basic metabolism and toxicity of AlOH. In the present work the rate of metabolism of AlOH and the accompanying changes in the hepatic reduced glutathione (GSH) content were related to formation of liver injury, as GSH is known to protect cellular functions *in vitro* by conjugating with acrolein (Rees & Tarlow 1967; Ohno *et al.* 1985). In addition, the significance of the *in vivo* detoxication of acrolein via aldehyde dehydrogenase was evaluated.

Materials and Methods

Animals and treatments. Male rats of the Alko mixed strain, aged 6–10 weeks, had free access to tap water and were deprived of food (R3, Ewos Ab, Sweden) overnight and during the experiment. A dose of 50 μl/kg (0.73 mmol/kg) of AlOH (0.25%, v/v; in H_2O) or 3.0 g/kg of ethanol (EtOH; 15%, w/v) or a mixture of both alcohols was intubated to rats via gastric tube between 7.30 and 11.30 a.m. In some experiments, including the dose of 75 μl/kg, 2% (v/v) AlOH was given. Neutralized 0.1% (w/v) disodium cyanamide (Fluka AG, Switzerland) in saline, corresponding to a dose of 1, 2 or 5 mg/kg, was injected intraperitoneally 1 hr prior to AlOH. Neutralized 100 mM 4-methylpyrazole hydrochloride (4-MP; Labkemi Ab, Sweden) in saline was given intraperitoneally (0.5 mmol/kg) 15 min. before AlOH.

Sampling. The rats were anaesthetized 10 min. before sampling (9.30 a.m.–2.30 p.m.) with 1% sodium pentobarbital in saline (60 mg/kg, intraperitoneally). The chest was opened and blood drawn from the right ventricle was transferred into a capped tube containing EDTA. 250 μl of blood was added to an equal volume of H_2O in a gas chromatography vial, that was sealed immediately and used for determination of alcohols. Blood diluted with saline was centrifuged for 15 min. at $1000 \times g_{max}$, and the plasma was stored at −75°.

A liver sample excised from the left main lobe was blotted with tissue. One piece was fixed in phosphate-buffered 10% (v/v) formalin (pH 7) for histological investigation. Another piece was immediately frozen in liquid nitrogen, stored at −75° and used for the GSH assay.

Analytical methods. The alcohols were assayed using a headspace gas chromatograph equipped with 2 m × 1/8 in. I.D. 60/80 Carbopak B/5% Carbowax® 20M stainless steel column (Supelco, Inc., U.S.A.). The temperatures of the headspace sampler, oven and FI-detector were 65, 75 and 140°, respectively. With the carrier gas flow rates used, the retention times for EtOH and AlOH were 1.3 and 3.5 min., respectively. The detection limit for AlOH in samples diluted to 500 μl with H_2O was 2 μM for heart blood (250 μl), and 5 μM for tail blood (100 μl). Recovery of added AlOH from blood was 94%.

Standard methods were used to assay the plasma activities of alanine aminotransferase (EC 2.6.1.2; Scandinavian Committee on Enzymes 1974), glutamate dehydrogenase (EC 1.4.1.2; Schmidt 1970) and γ-glutamyl transpeptidase (EC 2.3.2.2; Scandinavian Committee on Enzymes 1976).

The frozen liver samples were homogenized in 10 volumes of 10% (w/v) trichloroacetic acid – 12 mM Na_2-EDTA, and the non-protein sulfhydryl content in the supernatant (15 min. at $1000 \times g_{max}$) was assayed using Ellman's reagent (Ellman 1959). The results were in good agreement with those obtained by the fluorometric method (Hissin & Hilf 1976), which gave on average 15% lower values.

The formaline-fixed liver tissue was processed in the usual manner for routine paraffin embedding. The 5 μm thick preparates were stained with haematoxylin and eosin as well as with chromotrope aniline blue.

Statistics. Significances were calculated using one-way analysis of variance combined with Student-Newman-Keuls test.

Results

AlOH concentration in blood.

Seven min. after intragastric administration of AlOH (50 μl/kg) to rats the tail tip blood from only 3 of 18 rats contained detectable amounts (10±2 μM, S.E.M.) of AlOH. Less than 5 μM was found in heart punction blood at 12 min. in 3 of 5 animals. No AlOH was observed in blood collected from tail tip 10–30 min. after intubation. Based on complete disappearance of AlOH within 12 min. and on the liver weight being 3.6% of the body weight, a minimum hepatic capacity for AlOH oxidation of 1.7 μmol/(min. · g liver) was calculated. In blood from 4-MP-treated rats high concentrations of AlOH were still present 1 hr after administration (fig. 2).

Hepatic glutathione.

The hepatic content of non-protein sulfhydryl (mostly GSH) declined by about two thirds within 12 min. after the dosage of AlOH (fig. 1). This rapid initial depletion was followed by replenishment at a constant rate of about 1.25 μmol/(g · hr), so that at 5 hrs the content significantly exceeded the control range (fig. 1). Cyanamide pretreatment had no effect on the extent of this GSH rebound. Pretreatment of the animals with 4-MP, on the other hand, efficiently prevented the GSH depletion by AlOH (fig. 1).

Although the total amount of acrolein generated via liver alcohol dehydrogenase from 50 μl/kg of AlOH is approximately 3–4 times that of the hepatic GSH pool (~20 μmol/g liver versus 5–6 μmol/g liver), the hepatic GSH is not completely exhausted. The remaining GSH pool, representing about 30% of the initial, is, however, able to conjugate with acrolein, since with a double dose of AlOH over 90% depletion of hepatic GSH is observed (fig. 2).

Plasma enzymes.

Plasma samples collected 12, 30 and 60 min. after AlOH intubation contained normal or only slightly elevated alanine aminotransferase, γ-glutamyl transpeptidase and glutamate dehydrogenase activities. Within 3 hrs all three activities had increased moderately, but only after 5 hrs the elevations were remarkable indicating severe liver injury (table 1). The relative elevation was greatest for mitochondrial glutamate dehydrogenase (35-fold), while the activity of γ-glutamyl transpeptidase, which is low in rat liver, rose about four-fold. 4-MP efficiently prevented the release of the enzymes (data not shown).

Pretreatment of the rats with the aldehyde dehydrogenase inhibitor cyanamide before AlOH intubation caused a further moderate elevation of alanine aminotransferase, but had no effect on the two other enzyme activities (table 1).

Histology.

The initial stages of liver damage were visible in histopathological examination of samples taken five hours after admin-

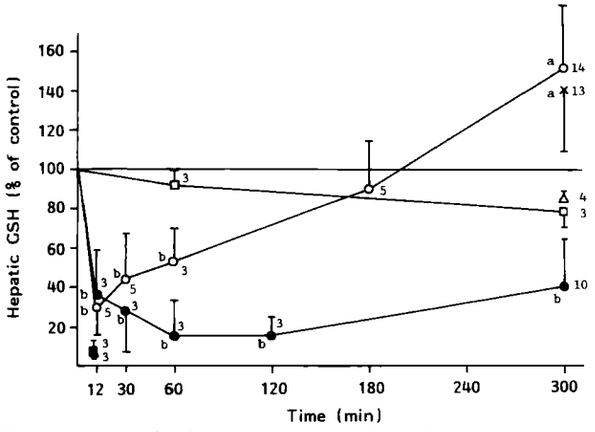

Fig. 1. Content of reduced glutathione in liver after administration of allyl alcohol and ethanol. GSH was assayed from liver TCA extracts of rats given AlOH (○) after pretreatment with 4-methyl pyrazole (□) or cyanamide (×) or given EtOH alone (△) or in combination with AlOH (●). In some experiments 100 μl/kg of AlOH was intubated to naive (▲) and cyanamide-pretreated (■) rats, livers were freeze-clamped 10–12 min. later and processed for GSH assay. The mean±S.D. is 5.18±0.80 μmol/g for untreated and cyanamide-treated controls (n=21). Means±S.D. and the number of animals are given. $^{a}P<0.01$ and $^{b}P<0.001$ in comparison with controls.

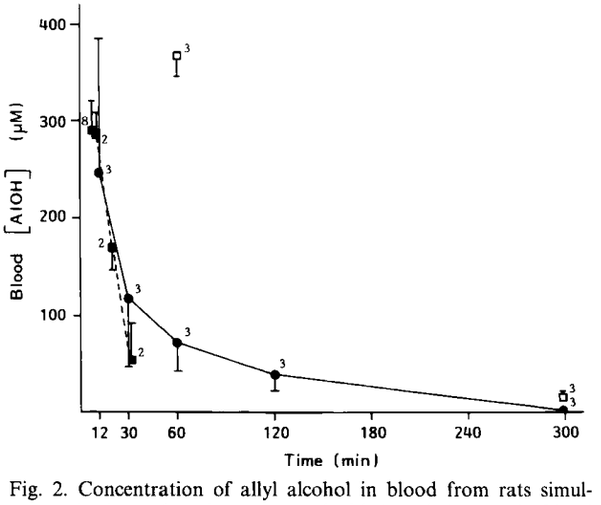

Fig. 2. Concentration of allyl alcohol in blood from rats simultaneously given ethanol or pretreated with 4-methyl pyrazole. Blood was collected from tail tip (■) or by cardiac punction (●) and assayed for AlOH by head-space gas chromatography. 4-MP (0.5 mmol/kg) in saline was injected intraperitoneally 15 min. before AlOH intubation (□). After co-administration of the alcohols EtOH concentrations ranging at 25–69 mM were present in blood from 7 min. to 5 hrs. Means±S.E.M. and the number of animals are given.

Table 1.

Plasma enzyme activities 5 hrs after treatment with allyl alcohol.

Treatment	n	Enzyme activity nmol/(min. · ml) Alanine aminotransferase	γ-Glutamyl transpeptidase	Glutamate dehydrogenase
None or cyanamide (a)	21	30 ± 2	2.8 ± 0.2	7 ± 1
Allyl alcohol (b)	14	406 ± 54^{aa}	12.8 ± 2.3^{aa}	235 ± 38^{aa}
Cyanamide + allyl alcohol§ (c)	13	$672 \pm 107^{aa,b}$	13.2 ± 1.6^{aa}	295 ± 24^{aa}
Ethanol + allyl alcohol	10	$134 \pm 46^{b,cc}$	$5.0 \pm 0.7^{b,c}$	$49 \pm 17^{bb,cc}$
Ethanol	4	25 ± 2	4.0 ± 0.4	3 ± 1

Enzyme activities were assayed from blood samples obtained by cardiac punction five hours after gastric intubation of allyl alcohol (50 µl/kg) or ethanol (3 g/kg). Cyanamide (1–5 mg/kg) was administered 1 hr prior to allyl alcohol. Significances corresponding $P < 0.01$ and $P < 0.001$ are indicated by one or two letters, respectively, referring to the similarly coded treatment group. Means ± S.E.M. are given.
§ One rat died and was excluded from the assays.

istration of AlOH. Histologic changes indicative of moderate or severe liver damage were seen in 22 of 37 rats treated with 50 µl (n = 27) or 75 µl (n = 10) of AlOH, and were located primarily in the periportal region of the acini. The other 15 animals exhibited only minimal changes located evenly along the acinus or were normal (table 2). The histologic appearance of livers from rats treated with cyanamide or EtOH only was similar to controls.

Treatment of the rats with cyanamide prior to AlOH administration did not elicit any further change in the pattern of histologically observable damage. However, the dose of AlOH seemed to influence the degree of injury observed at 5 hrs (table 2).

Effect of ethanol on allyl alcohol toxicity.

Administration of EtOH together with AlOH significantly suppressed the elevation of the plasma enzymes (table 1). Although occasional elevations occurred, the means did not differ significantly from controls. Co-administration of EtOH also completely prevented the histologically observable signs of liver damage occuring in the majority of rats receiving only AlOH (table 2). However, EtOH did not have an effect on the initial depletion of hepatic GSH by AlOH, but it prolonged the depletion to last for at least 5 hrs (fig. 1).

Concomitantly with these effects, EtOH also markedly decelerated the oxidation of AlOH. The concentration of AlOH in the blood was about 0.3 mM at 10 min., and its complete disappearance required more than 2 hrs (fig. 2). Since the inhibition by EtOH of AlOH oxidation via alcohol dehydrogenase is competitive, the molar excess of EtOH, and hence the inhibition, will increase as the AlOH concentration decreases. In experiments with isolated hepatocytes we found that a 100-fold molar excess of EtOH inhibited the rate of oxidation of 0.4 mM AlOH by 55%. Ten minutes after administration of both alcohols their blood concentration ratio was about 150. The oxidation rate of AlOH in liver during the 12–30 min. and 60–120 min. period can be estimated from fig. 2 to be about 0.15 and 0.01 µmol/(g · min.), respectively. The calculated oxidation rate of AlOH during the initial phase indicates an inhibition of over 90%. This is conceivable with the estimate obtained from the cell incubation experiments by extrapolating to the proper EtOH/AlOH ratio.

Table 2.

Histologically observable liver injury in rats five hours after administration of allyl alcohol.

Treatment		n		Degree of injury[a] 0		1		2	
None		9		4		5			
Na-cyanamide		6	19	3	53%	3	47%		0%
Ethanol		4		3		1			
Ethanol + allyl alcohol	50 µl/kg		8	5	62%	3	38%		0%
Allyl alcohol	50 µl/kg	13	17		0%	6	41%	7	59%
	75 µl/kg	4				1		3	
Na-cyanamide + allyl alcohol	50 µl/kg	14	20	2	10%	5	30%	7	60%
	75 µl/kg	6				1		5	

Number of cases exhibiting the indicated degree of injury is shown. Combined values of n correspond 100%. Treatment of animals was as in table 1.
[a]Arbitrary classification: 0, normal, no changes; 1, minimal changes: minimal fatty changes, when only occasional fat droplets were seen. Some small lymphocyte accumulation was also noticed in the liver parenchyma of untreated animals; 2, moderate or severe injury: necroses and haemorrhages especially periportally, in severe cases also pericentrally with portal-portal and/or portal-central bridging.

Discussion

Administration of 50 μl/kg of AlOH to fasted rats led to severe hepatic injury observable already after 5 hrs, as demonstrated by the release of intracellular enzymes into circulation and formation of primarily prenecrotic tissue changes seen in histopathological examination of the liver. The damage, evaluated on the same criteria, has been reported to reach its maximal intensity 12–24 h after AlOH administration (Thorgeirsson *et al.* 1976; Gumucio *et al.* 1978; Hanson & Anders 1978; Gumbrecht & Franklin 1983).

AlOH given via gastric tube was rapidly absorbed and oxidized by liver alcohol dehydrogenase, since only traces of AlOH were occasionally present in blood 10 min. after administration. This finding is in accordance with that of Poulsen & Korsholm (1984), who reported AlOH to be rapidly eliminated after an oral dose of 100 μl/kg. In contrast, Belinsky *et al.* (1984a) reported that 30 min. after injecting 50 μl/kg of AlOH intraperitoneally its concentration in venous blood still was 0.5 mM. The discrepancy between these results can hardly be explained only by different routes of administration. The minimum oxidation rate for AlOH in liver calculated by us (1.7 μmol/(min. · g)) is somewhat higher than that reported by Belinsky *et al.* (1984a & b) from perfusion studies.

The rapid loss of hepatic GSH during the oxidation of AlOH is obviously due to conjugation with acrolein. With regard to the molar excess of acrolein, it is probable, that a large fraction of acrolein will bind covalently to other biomolecules and initiate irreversible cellular injury very soon after AlOH administration. However, with the indicators used, the signs of the injury are manifested only several hours later. The failure of Poulsen & Korsholm (1984) to detect GSH depletion by AlOH can be explained by the 6-hour latency between AlOH administration and the first moment of sampling.

Considering the high rate of formation of acrolein, and its high reactivity with GSH, a complete depletion of GSH by 50 μl/kg of AlOH could be expected. However, about one third of the hepatic GSH still remains. On the other hand, a more complete depletion results from a double dose of AlOH (100 μl/kg). This raises the possibility that AlOH oxidation and GSH depletion occur non-uniformly in the acinus, so that more AlOH is oxidized in the periportal region. Depletion of GSH would be total in this region, while the perivenous region would be much less affected. Only when the dose, and hence the portal blood concentration, of AlOH is increased, the acinar zone of AlOH oxidation and extensive GSH depletion would extend towards the perivenous end of the sinusoid.

The initial fall of GSH gave rise to immediate onset of GSH resynthesis. This led to over-compensation of the GSH loss within five hours. However, after AlOH treatment the GSH content in different acinar regions may vary a lot, and locally it may exceed the mean hepatic GSH content displayed in fig. 1. Obviously the mean hepatic GSH remains elevated during the formation of necrosis and at least for 24 hrs (Atzori *et al.* 1980; Poulsen & Korsholm 1984), although the extent of elevation may be age-dependent (Rikans & Kosanke 1984).

Liver soluble and microsomal aldehyde dehydrogenase can oxidize acrolein to acrylic acid (Patel *et al.* 1980), but the role of this potential detoxication reaction for acrolein is not established. Ohno *et al.* (1985) found that inhibition of aldehyde dehydrogenase with disulfiram potentiated the toxicity of AlOH in isolated hepatocytes. In this work inhibition of aldehyde dehydrogenase by cyanamide prior to AlOH administration potentiated slightly the elevation of one (ALAT) of the three enzymes, but had no histologically observable effect nor did it effect the extent of the GSH rebound. These results do not indicate a major role for aldehyde dehydrogenase in detoxication of AlOH-derived acrolein *in vivo*.

The protection by EtOH against AlOH-induced liver damage demonstrated by this study and also seen in a short-term perfusion study (Schwarzmann *et al.* 1967) is attributed to the competitive inhibition by EtOH of acrolein formation via alcohol dehydrogenase. Patel *et al.* (1983), using deuterium-labeled AlOH, have also demonstrated the interdependence of the oxidation rate of AlOH and the extent of toxicity as well as binding of the label to hepatic proteins.

Although EtOH inhibited AlOH oxidation, GSH was depleted within 12 min. to the same extent as with AlOH alone. This shows that in the presence of EtOH acrolein production still saturates, but does not massively exceed, the capacity of the GSH pool for immediate conjugation. Thus, a major fraction of acrolein is detoxified. Although it is not possible to obtain the actual rate of GSH depletion from the present data, the average initial rate of AlOH oxidation in the presence of EtOH and of GSH depletion can be approximated to be roughly equal, 0.15–0.3 μmol/(g · min.). After 12 min. the GSH content remains depleted as acrolein is continuously produced at a rate exceeding the rate of GSH synthesis.

After 1 hr following simultaneous administration of the alcohols new GSH is presumably synthesized at a rate sufficient to detoxify the acrolein generated. The GSH content seems to increase only after all AlOH has been oxidized. EtOH may also prolong the GSH depletion by direct inhibition of the synthesis of GSH (Lauterburg *et al.* 1984; Speisky *et al.* 1985).

In summary, acrolein, that is produced from a sublethal dose of AlOH possibly mainly in periportal hepatocytes, seems not to be appreciably detoxified via oxidation by hepatic aldehyde dehydrogenase, but mostly reacts with GSH and other molecules leading to liver damage after a latency period of 4–5 hours. EtOH protects liver against injury by competing with the alcohol dehydrogenase-catalyzed oxidation of AlOH, but prolongs the depletion of hepatic GSH due to continuous production of acrolein. Thus, the liver can withstand a long-lasting depletion of GSH, as well as moderate amounts of AlOH-derived acrolein produced at relatively low rate, without showing signs of serious cellular damage. More promptly, the basic determi-

nant of AlOH hepatotoxicity appears to be the rapid rate of acrolein formation and the amount of acrolein escaping conjugation with GSH.

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