EFFECT OF VERAPAMIL ON ALLYL ALCOHOL HEPATOTOXICITY

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SUMMARY

The effects of verapamil, a calcium channel blocker, on allyl alcohol (AA) hepatotoxicity were studied *in vivo*. AA administration induced an increase of serum alanine aminotransferase (ALT) concentration and liver necrosis by means of glutathione (GSH) depletion. Pretreatment with verapamil reduced the increase of ALT in plasma and the morphological signs of necrosis induced by AA administration. Verapamil did not affect GSH levels by itself but prevented the decrease of the tripeptide by AA. *In vitro*, but not *in vivo*, verapamil inhibited the activity of alcohol dehydrogenase (ADH), the key enzyme in the conversion of AA into the toxic metabolite acrolein. These data indicate that verapamil protects against AA toxicity, probably by preventing the production of acrolein, its reactive metabolite.

KEY WORDS

allyl alcohol, verapamil, hepatotoxicity, alcohol dehydrogenase, glutathione

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INTRODUCTION

Allyl alcohol (AA) is a potent hepatotoxin that, in contrast to most other hepatotoxins, induces necrotic injury in the periportal regions of the liver acinus /1/. It is metabolized via the cytosolic enzyme alcohol dehydrogenase (ADH) to the corresponding aldehyde, acrolein, with no involvement of cytochrome P-450-dependent mixed function oxidase in the mechanism of toxicity /2-4/. Although the mechanism by which AA induces hepatotoxicity is unclear, it is accepted that it is dependent on its metabolism to acrolein. This α,β-unsaturated aldehyde is a powerful electrophile that readily forms an adduct with sulfhydryl groups of cellular constituents, especially glutathione (GSH), and thereby inactivates essential cellular macromolecules /5-7/. The main routes of AA detoxification are the inhibition of its conversion by ADH and the conjugation of acrolein to GSH or sulfhydryl compounds /3,8/. Accordingly, depletion of cellular GSH has been shown to potentiate the toxicity of AA, whereas increasing the available sulfhydryl groups is protective /5/.

It has been suggested that the alkylation of nucleophilic groups of cellular macromolecules affected by acrolein after glutathione depletion is the event which leads to cell injury /3,8/. Recent reports have suggested that a major role in the production of AA-induced hepatotoxicity might be played by lipid peroxidation /9-11/, as in the case of other GSH-depleting agents /12/. On the other hand some reports excluded an involvement of lipid peroxidation in AA hepatotoxicity /13-14/. Therefore peroxidative damage of lipids might be just a consequence of the cytotoxicity of AA and acrolein induced by the covalent binding of acrolein to cellular macromolecules as in other models /15/.

Several mechanisms of protection against AA intoxication have been proposed, such as pretreatment with pyrazole or methylpyrazole, inhibitors of ADH, and administration of N-acetylcysteine or other GSH precursors; the role of antioxidants (e.g. butylated hydroxytoluene, Trolox, desferrioxamine) is still controversial /3,8/.

Many investigators have postulated that a perturbation in calcium homeostasis may represent an early event and final common pathway leading to cell death in many types of cell injury /16/. Depletion of GSH stores has been associated with elevated cytosolic calcium content /17/. Inasmuch as GSH depletion by some other chemicals (e.g. paracetamol, bromobenzene, cystamine) and in liver injury induced by non-chemical means (e.g. ischemia/reperfusion) is linked to elevated

cytosolic calcium, it has been suggested that the periportal hepatotoxicity of AA could involve the same mechanism /18/. Although the participation of calcium ions in AA intoxication is still debated, it has been suggested that the alteration of hepatocellular calcium homeostasis is produced by the loss of soluble protein-bound thiols secondary to GSH depletion /18/. The role of extracellular calcium in the induction of cell death by toxins in the liver is subject to debate /19/.

Recently, much attention has been focused on the role of calcium channel blockers in the prevention of oxidative injury. Since Ca^{2+}-entry blockers have been shown to be protective in several experiments /20,21/, the purpose of the present study was to investigate the effect of verapamil, a well known calcium channel blocker, as a potential agent in the prevention of AA hepatotoxicity in rats.

MATERIALS AND METHODS

Materials

Allyl alcohol was obtained from Merck (Germany). All other chemicals were purchased from Sigma (Italy). To measure serum alanine aminotransferase (ALT) activity a commercially available kit (Boerhinger-Mannheim, Germany) was used.

Animals and treatment

Male Sprague-Dawley rats weighing 250-350 g were obtained from Charles River (Italy). The animals were allowed free access to water and standard rat chow (Piccioni, Italy). They were housed in groups on hardwood bedding under constant environmental conditions.

Rats received AA (50 μl/kg) per os diluted in saline or an equivalent volume of saline. In order to evaluate the effect of verapamil rats were treated with 20 μmoles/kg per os of verapamil 15 min before exposure to AA. Controls received a similar volume of solvent. Animals were sacrificed 60 min and 24 hours after AA treatment.

Preparation of ADH liver fraction

Animals treated with verapamil, 20 μmoles/kg, and controls were sacrificed by excess sodium pentobarbital. The liver was washed with 0.25 M sucrose solution, minced and homogenized in 0.25 M sucrose (10% vol/weight). The supernatants were obtained by centrifugation at

12,500 *g* for 60 min at 4°C. Activity of ADH in the supernatant was determined by the method of Bonnichsen and Brink /22/ by monitoring NADH production as the increase in absorbance at 340 nm in a Perkin-Elmer 550S spectrophotometer. In the experiments with purified ADH, ethanol was used as substrate and the enzyme activity was determined in the presence or absence of verapamil and pyrazole. The amount of protein in the above experiments was measured according to Paterson using bovine serum albumin as a standard /23/.

Histopathology

The effects of allyl alcohol and verapamil on hepatic morphology *in vivo* were assessed by light microscopy. Rats were anesthetized with sodium pentobarbital. Aliquots of the liver were fixed in Bouin's solution, then embedded in paraffin and processed for light microscopy. Sections were stained with hematoxylin-eosin.

Measurement of ALT

Levels of ALT in serum were measured spectrophotometrically using a standard assay (Boehringer Ingelheim, Germany). Values were expressed as U/l.

Measurement of GSH

Frozen liver samples were homogenized in 10 volumes of 10% (w/v) trichloroacetic acid, and after centrifugation the non-protein sulfhydryl content in the supernatant was assayed using Ellman's reagent /24/.

Statistics

Results are given as means ± S.E.M. Data were analyzed by Student's t-test, using a standard, computerized statistical program (INSTAT by GraphPadD, Sigma). Differences with $p<0.05$ were considered significant.

RESULTS

The intragastric administration of AA (50 µl/kg) resulted in an increase of ALT hepatic enzyme activities in plasma 24 h later (Fig. 1).

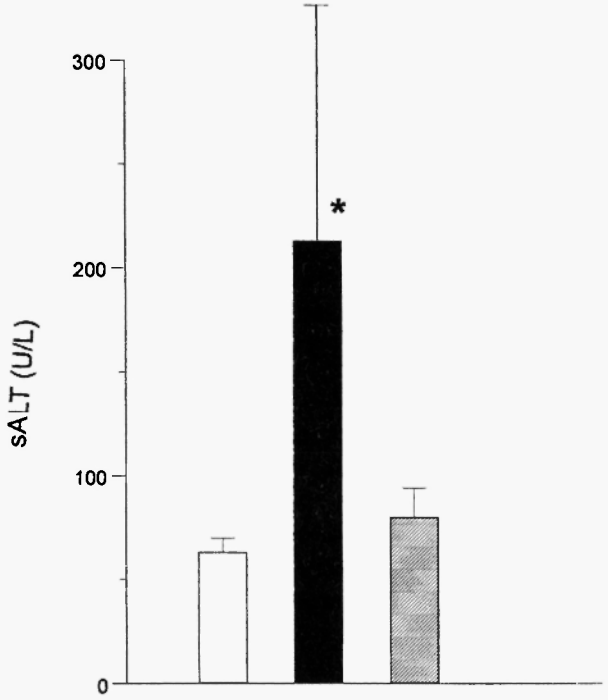
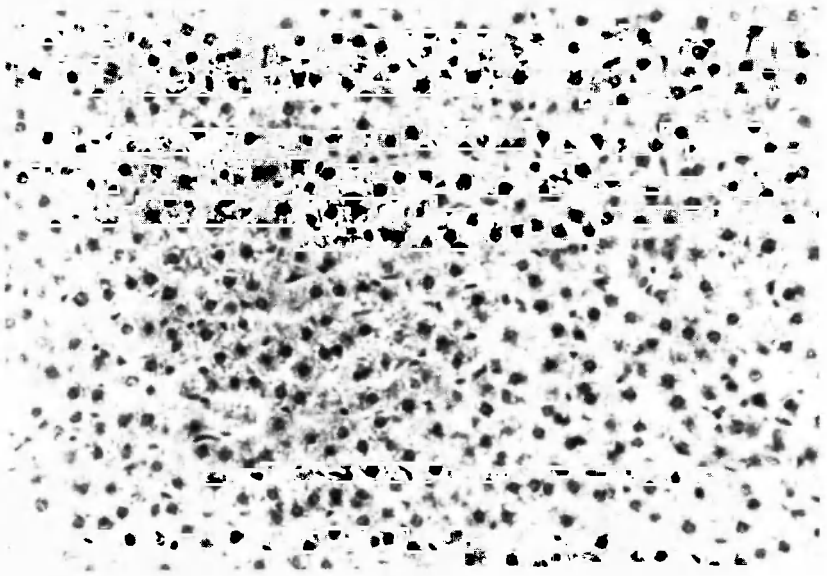


Fig. 1: Serum ALT in control and verapamil treated rats 24 h after exposure to AA. Rats were pretreated with verapamil (20 μmoles/kg, per os) 15 min prior to administration of AA (50 μl/kg, per os). Values are means ± S.E.M. of four to five rats in each treatment group. Control (□); AA (■); verapamil + AA (▨). *$p<0.05$ AA vs control and AA vs verapamil + AA.

This indicator of liver necrosis was accompanied by histopathologically observed changes such as periportal foci of necrotic hepatocytes (Fig. 2). In some preparations a marked acute inflammatory response consisting of polymorphonuclear infiltration was present within and at the margin of this necrotic zone. When the rats were pretreated with verapamil, 20 μmoles/kg 15 min before AA administration, the levels of sALT were significantly lower when compared to AA alone after 24 h (Fig. 1). Pretreatment with verapamil also prevented signs of necrosis in the liver (Fig. 2). Due to the critical role of GSH in AA-induced hepatotoxicity the levels of this tripeptide in the liver were studied. One hour after AA treatment a significant depletion in hepatic GSH content was observed (Fig. 3), but in the groups of verapamil alone or



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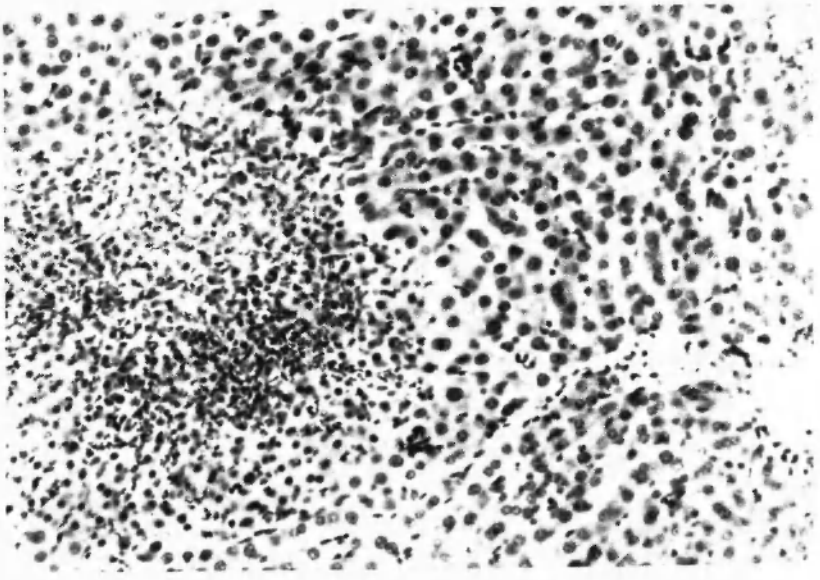

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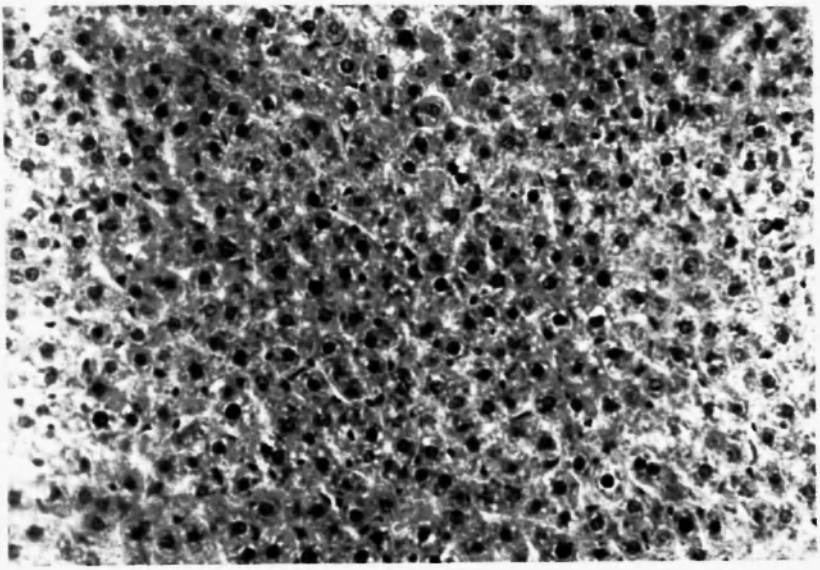

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Fig. 2: Rat liver control (a) or 24 h after treatment with AA (50 µl/kg, per os) (b) or verapamil (20 µmoles/kg, per os) plus AA (c). In b marked signs of necrosis and inflammation are evident. H & H (x80).

plus AA the values were not different from the controls (Fig. 3). After 24 h the levels of GSH were similar in all groups (Fig. 3).

As alcohol dehydrogenase plays a critical role in the metabolism of AA by converting the alcohol into the toxic derivative acrolein, a possible effect of verapamil on the enzyme was investigated. Livers from rats treated with verapamil were homogenized and ADH activity evaluated. No differences from controls were observed (Fig. 4). Since the lack of effect *in vivo* by verapamil on ADH could be explained by the low levels of the enzyme in our crude preparation and/or to a specific distribution of the enzyme in hepatic cells, the effect of verapamil on ADH activity was studied *in vitro* using purified enzyme and ethanol as substrate. Pyrazole, a well known ADH inhibitor, was used as positive control. Addition of verapamil to the ethanol and ADH mixture inhibited the enzyme activity, similarly to pyrazole (Fig. 5).

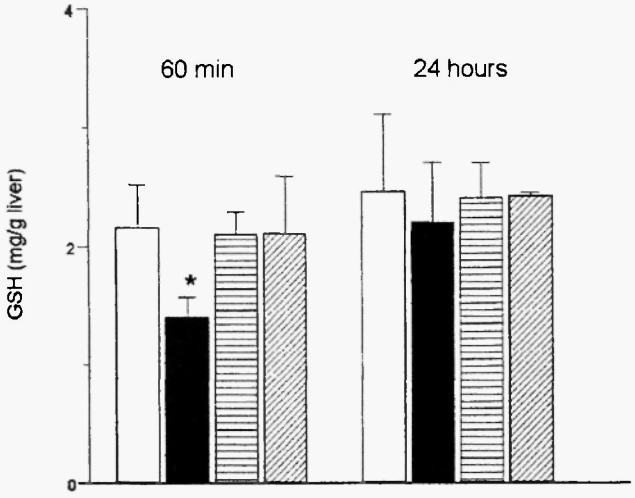

Fig. 3: Hepatic GSH content in control and verapamil treated rats 60 min and 24 h after exposure to AA. Rats were pretreated with verapamil (20 μmoles/kg, per os) 15 min prior to administration of AA (50 μl/kg, per os). Values are means + S.E.M. of four to five livers in each treatment group. Control (□); AA (■); verapamil (⊟); verapamil + AA (☑). *$p<0.05$ AA vs control and AA vs verapamil + AA.

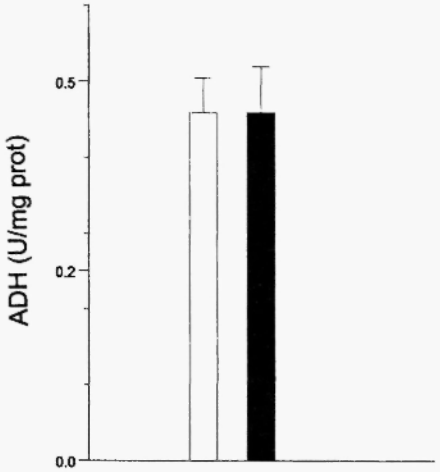

Fig. 4: ADH activity in liver homogenate of verapamil treated rats after 75 min. Rats were treated with verapamil (20 μmoles/kg, per os) and 75 min later were sacrificed. ADH activity was measured in the liver homogenate and compared to control. Control (□), verapamil (■). Values are means ± S.E.M.

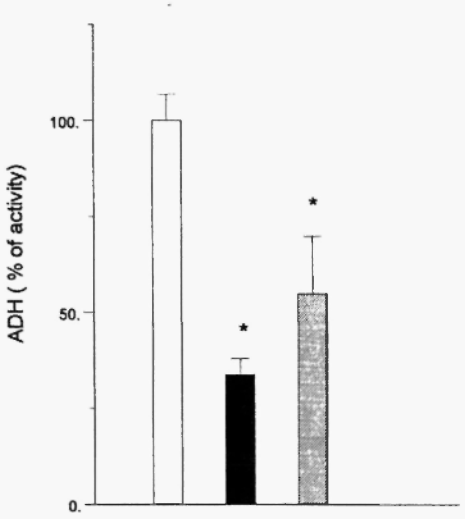

Fig. 5: Activity of purified ADH after addition of 11 mM verapamil (■) or 7 mM pyrazole (▨). Values are expressed as percent changes in activity with respect to control (□) and are means + S.E.M. *$p<0.01$ verapamil vs control and pyrazole vs control.

DISCUSSION

The results of the present investigation show that verapamil, a Ca^{2+} channel blocker, affords protection against AA hepatotoxicity, probably by inhibiting the conversion of AA into the reactive metabolite acrolein via ADH. Conversion of AA into acrolein is a critical stem in the toxicity of this compound /2-4/. The formation of the aldehyde gives rise to a cascade of events leading to cell necrosis. Decreased levels of GSH, or sulfydryl groups in general, have been shown to be critical in AA toxicity and thiol-containing compounds have been shown to protect against AA-induced hepatoxicity /3-8/. AA administration was associated with severe depletion of GSH *in vivo* and in isolated hepatocytes. Indeed it has been demonstrated that pretreatment with GSH depletors potentiates AA toxicity /25/. In our experimental conditions verapamil did not affect the levels of GSH, but prevented the changes induced by AA. This observation supports an inhibitory effect of verapamil on ADH as the protective mechanism.

Excessive influx of calcium ions has been associated with cell death by activating many enzymatic processes, including phospholipases, proteases and endonucleases /26/. The alteration of Ca^{2+} homeostasis may be produced by the loss of soluble and protein-bound thiols secondary to GSH depletion /26/. Since sulfhydryl groups are critical in the control of intracellular Ca^{2+} levels, modification of sulfhydryl groups can result in changes in Ca^{2+} homeostasis /26/. Although perturbation of Ca^{2+} homeostasis is believed to be important in the mechanism of cell death, in the case of AA-hepatotoxicity a clear involvement of Ca^{2+} has not been proved /27/.

It has been previously shown that some effects of verapamil might not be mediated by Ca^{2+} channel inhibition. Effects of verapamil apart from the Ca^{2+} channel block include changes in GSH levels /28/, an inhibitory effect on lipid peroxidation /29/, inhibition of the P-450-dependent biotransformation of drugs /30/, and others /31/. Many of these non-specific cellular effects of calcium antagonists could possibly protect the hepatocytes without affecting calcium channels. Moreover the beneficial effects of calcium antagonists in the liver may occur in cells other than hepatocytes since it is generally accepted that these cells do not possess voltage sensitive calcium channels /31/.

Although verapamil injection at a dose higher than the one we used decreased hepatic GSH content /28/, our dosage did not induce a depletion of GSH.

Following treatment with AA we did not observe an increase in hepatic lipid peroxidation /3,5/, so the antioxidant properties of verapamil do not apply in this case /21/. This could be explained by differences in the species used, mouse vs rat, and the route of AA administration.

In conclusion, this study shows that verapamil may influence ADH activity and prevent AA toxicity, which occurs via its conversion by ADH into acrolein /3-4,8/. Since the results in rats do not necessarily apply to humans, clinical significance must be confirmed before using verapamil to treat alcohol intoxication in man.

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