Hepatotoxicity of alcohol-induced polar retinol metabolites involves apoptosis via loss of mitochondrial membrane potential

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SPECIFIC AIMS

The aims of our study were to 1) establish whether the enhanced vitamin A hepatotoxicity in alcoholics could be related to toxic polar retinol metabolites (PRM) generated by alcohol-induced microsomal enzymes and 2) assess the underlying mechanism of cell death.

PRINCIPAL FINDINGS

1. Identification and purification of polar retinol metabolites

Chronic alcohol consumption interferes with vitamin A metabolism and may lead to a striking vitamin A depletion due to a competitive antagonism for the same hepatic enzymes alcohol dehydrogenase (ADH) and cytochrome P4502E1 (CYP2E1). Vitamin A supplementation may correct this deficiency, but chronic alcohol consumption enhances the intrinsic hepatotoxicity of vitamin A. We have recently demonstrated in rat that ROH and RA are biotransformed by induced CYP2E1 into the PRMs 18-OH-RA and 4-oxo-RA, while the selective inhibition of CYP2E1 by chlormethiazole prevents PRM production.

For our experiments, PRMs extracted from livers of alcohol- and control-fed male Sprague-Dawley rats were used. PRMs were identified and extracted by a gradient reverse-phase HPLC in which RA and ROH eluted at 20.5 min and 41.8 min, respectively. Several metabolites (peaks indicated as polar metabolites in Fig. 1) were detected between RA and ROH in the liver of ethanol-fed rats and collected by HPLC. HPLC analysis showed that PRMs from livers of ethanol-fed rats comprise at least four components (Fig. 1B) not detectable in control extracts (Fig. 1A). PRM concentration was estimated based on the HPLC peak of the internal standard retinyl acetate. PRM and control extracts were serially diluted to 0.3–0.005 μmol/L with DMEM.

2. Effect of PRMs on cell viability and nuclear morphology

For the cell culture experiments, HepG2 cells and freshly isolated rat hepatocytes were used. After treatment with PRM or control extracts, HepG2 cells and hepatocytes were stained with trypan blue and visualized under a light microscope. PRM caused marked morphological changes in both HepG2 cells and isolated hepatocytes. The membrane shape was irregular and revealed membrane blebbing and cytoplasmic shrinkage. Cells were dispersed and the monolayer was distorted. No such changes in morphology were evident in cells treated with the control extract. PRM were cytotoxic in a dose- and time-dependent manner in HepG2 cells and primary hepatocytes as assessed by trypan blue staining. Treatment with 0.3 μM of PRM in HepG2 cells and 0.15 μM of PRM in primary hepatocytes caused an equivalent loss of cell viability after 3 h reaching ~75% after 24 h. Cell viability was not affected by the control extract.

As a surrogate marker of direct cytotoxicity from PRM, LDH/AST activities were determined from cell lysates. LDH and AST leakage from HepG2 cells due to PRM occurred dose- and time-dependently after 3–24 h treatment with PRM concentrations of 0.02–0.3 μmol/L. PRM at 0.3 μmol/L induced a rapid leakage of LDH at 3 h that gradually rose over time, reaching 89% of total LDH activity after 24 h. Lower concentrations of PRM (≤0.02 μmol/L) and the control extract did not induce LDH release. Treatment with PRM induced AST elevations in the same proportion as for LDH. PRM at 0.3

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µmol/L caused a 10-fold increase of AST release at 24 h when compared with controls.

For assessment of nuclear morphology, treated cells were fixed with methanol/acetic acid and stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI); we also used the DNA-dye AO (acridine orange) in vital cells. After PRM treatment of HepG2 cells, chromatin condensation and DNA fragmentation were detected with DAPI staining whereas the nuclei of untreated cells remained unaffected. With AO, nuclei of control cells showed chromatin with regular fluorescence, while PRM-treated cells revealed chromatin condensation and fragmentation.

3. PRMs are proapoptotic through damaging mitochondrial membrane potential

DNA fragmentation as a typical feature of apoptotic cell death was evaluated by FACS analysis using hypotonic PI (propidium iodine) solution that detects apoptotic cells by measuring the fraction of nuclei containing a hypodiploid DNA content (sub-G1 fraction). Here, exposure of HepG2 cells to PRM caused a marked increase of the sub-G1 at 48 h compared with control cells, reaching 20% at 120 h confirming PRM-induced apoptosis.

The impact of PRM on mitochondrial membrane potential (ΔΨm) was determined by FACS analysis using JC-1 (5,5,6,6-tetra-chloro-1,1,3,3-tetraethylbenzimidazol-carbocyanine iodide), which reflects changes of the ΔΨm as a fluorescence shift from orange to green, typically when the cell undergoes apoptosis. Figure 2 displays a marked time-dependent reduction of ΔΨm after exposure of HepG2 cells to PRM at 0.3 µmol/L with a decrease of orange fluorescence paralleled by an increased transition to green fluorescence. Changes occurred after 1 h and further intensified dramatically over 24 h. PRM treatment of primary hepatocytes also caused a similar dose-dependent loss of ΔΨm in hepatocytes, even with a PRM concentration as low as 0.04 µmol/L.

Within the apoptotic cascade, activation of caspases is a crucial event usually precipitated by the release of cytochrome C from mitochondria. Activation of caspases was determined by Western blot analysis using
antibodies that react exclusively with activated (cleaved) caspase-3 and -9. PRM caused a gradual and time-dependent increase of both cleaved caspase-9 and -3, which was marked between 12 and 24 h.

CONCLUSIONS AND SIGNIFICANCE
We show that PRMs extracted from livers of rats chronically fed alcohol cause cell death in HepG2 cells and freshly isolated primary rat hepatocytes. PRM-related cell death is driven by apoptosis, initiated by the loss of $\Delta \Psi m$ followed by caspase activation. PRM toxicity was further evidenced by a disruption of cell morphology, positive cellular staining with trypan blue, and the release of cytoplasmic enzymes into the cell culture medium.

Alcohol-induced liver damage occurs due to oxidative stress derived from reactive oxygen species (ROS) with subsequent lipid peroxidation, the toxicity of acetaldehyde, and the depletion of glutathione (GSH) and several micronutrients. Most pathomechanisms are closely linked to the up to 10-fold induction of CYP2E1, which is not only the main source of oxidative stress and acetaldehyde, but also metabolizes and activates many substrates into toxins. Alcohol-induced liver disease is partly driven by the induction of apoptosis through acetaldehyde and ROS that rapidly decrease mitochondrial GSH content and consequently cause a loss of mitochondrial function. GSH depletion leads to breakdown of the mitochondrial membrane potential, which is a central regulatory mechanism to maintain cell viability and in the onset of apoptosis.

In our study, PRM-induced apoptosis is initiated by a rapid breakdown of $\Delta \Psi m$ followed by the activation of caspase-9 and -3. Mitochondria are considered the key organelle in the initiation of apoptosis since mitochondrial disintegration not only leads to a loss of their function but also causes the release of proapoptotic factors, such as cytochrome C and apoptosis activating factor 1 (Apaf 1) triggering the caspase cascade. Caspase-9 subsequently activates effector caspases, such as caspase-3 (Fig. 3). The loss of $\Delta \Psi m$ in HepG2 cells occurred within 1 h, suggesting that mitochondrial depolarization is an early event of PRM-induced hepatocyte death. Disruption of $\Delta \Psi m$ preceded all other features of apoptosis, followed by caspase-9 and caspase-3 activation and finally DNA fragmentation.

Based on the fact that several retinoids including ROH and RA can be metabolized by liver microsomes, it was suggested that retinoid metabolites participate in alcohol-related enhanced vitamin A toxicity. Recently, we found that induction of CYP2E1 in alcohol-fed rats not only decrease hepatic ROH, but also leads to the formation of PRMs. However, the question whether these PRMs are hepatotoxic remained unanswered until now.

Administering vitamin A to alcoholics can be detrimental and treatment efforts must carefully account for the narrow therapeutic window of retinoids in alcoholics. Whether the administration of $\beta$-carotene, a provitamin A without supposed intrinsic hepatotoxicity, can overcome this therapeutic dilemma is unclear. A recent cancer prevention trial found that higher alcohol intake associates with more side effects of $\beta$-carotene supplementation. Previous data have demonstrated that $\beta$-carotene and concomitant alcohol ingestion cause liver damage, possibly due to synergistic induction of CYP2E1 and CYP4A1 by ethanol and $\beta$-carotene.

We have not identified the exact polar retinol metabolites due to the existence of multiple compounds and the lack of standards for unknown metabolites.

Our data provide a mechanistic explanation for the potentiation of vitamin A hepatotoxicity by alcohol and close the loop between the induction of CYP2E1, production of hepatotoxic RPM, and resulting liver damage.