Retinol/Ethanol Drug Interaction during Acute Alcohol Intoxication in Mice Involves Inhibition of Retinol Metabolism to Retinoic Acid by Alcohol Dehydrogenase*

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1Abbreviations: ADH, alcohol dehydrogenase; Adh1, mouse class I alcohol dehydrogenase gene; ALDH, aldehyde dehydrogenase; RA, all-trans-retinoic acid.

Running Title: Ethanol Inhibition of Retinol Metabolism to Retinoic Acid
SUMMARY

Substantial evidence indicates that one consequence of alcohol intoxication is a reduction in retinoic acid (RA) levels. Studies on the mechanism have shown that chronic ethanol consumption induces P450 enzymes that increase RA degradation, thus accounting for much but not all of the observed decrease in RA. A reduction in RA synthesis may also be involved as ethanol competitively inhibits retinol oxidation catalyzed by alcohol dehydrogenase (ADH) in vitro. This may be important during acute ethanol intoxication and may contribute to adverse retinol/ethanol drug interactions. Here we have examined mice for the effect of either acute ethanol intoxication or Adh1 gene disruption on RA synthesis and degradation. RA produced following a dose of retinol (50 mg/kg) was reduced 87% by pretreatment with an intoxicating dose of ethanol (3.5 g/kg). RA produced in Adh1 null mutant mice following a 50 mg/kg dose of retinol was reduced 82% relative to wild-type mice, thus similar to wild-type mice pretreated with ethanol. Reduced RA production was associated with increased retinol levels in both ethanol-treated wild-type mice and Adh1 null mutant mice, indicating reduced clearance of the retinol dose. RA degradation following a dose of RA (10 mg/kg) was increased only 42% by ethanol pretreatment (3.5 g/kg) and only 26% in Adh1 null mutant mice relative to wild-type mice. These findings demonstrate that reduced RA levels observed during acute retinol/ethanol drug interaction involves primarily a decrease in ADH-catalyzed RA synthesis plus secondarily an increase in RA degradation.

INTRODUCTION

Vitamin A (retinol) is metabolized to retinoic acid (RA) which serves as a ligand for nuclear retinoid receptors essential for growth and development of chordate animals (1,2). Retinoid signaling influences pattern formation during development of several organs including the central nervous system (3-5), limb buds (6-8), and eye (9). Also, RA controls epithelial/mesenchymal inductive interactions during organogenesis of the urinary and respiratory tracts (10-12), and RA is
needed in the adult to control epithelial cell differentiation (13) and to provide some brain functions such as spatial learning and memory (14,15) and motor skills (16).

Retinoid activation is performed by enzymes that first oxidize retinol to retinal, followed by oxidation of retinal to RA. Many of the retinoid-metabolizing enzymes identified *in vitro* are members of the same families of alcohol- and aldehyde-metabolizing dehydrogenases that oxidize ethanol to acetaldehyde and acetaldehyde to acetic acid, i.e. alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), respectively (17). Deactivation of RA by oxidation to more polar metabolites can be performed by various cytochrome P450 enzymes (18-20). Thus, the steady-state level of RA is dependent upon the activities of both synthesizing and degrading enzymes.

It has been reported that chronic ethanol treatment of rats leads to a reduction in RA levels in liver and serum, and that this may contribute to ethanol-induced liver carcinogenesis (21). Further studies on the mechanism have indicated that much, but not all, of the decrease in RA levels during chronic ethanol treatment can be attributed to an increase in RA degradation by ethanol-inducible CYP2E1, a P450 which was shown to metabolize RA to more polar metabolites (22). However, this may not be the case for acute ethanol treatment as it takes days to weeks for CYP2E1 activity to be induced by ethanol (23). Thus, the mechanism whereby ethanol reduces RA levels may be different depending on whether ethanol administration is acute or chronic.

It has been hypothesized that ethanol may reduce RA synthesis by acting as a competitive inhibitor of ADH-catalyzed retinol oxidation (24,25). Mouse embryos treated acutely with ethanol have reduced levels of RA which may be a contributing factor in the pathogenesis of fetal alcohol syndrome (26). Five classes of ADHs are known to exist in humans and mice (27). Studies on purified human ADH1 and ADH4 have shown that these enzymes are direct targets of ethanol action since ethanol can competitively inhibit their abilities to catalyze retinol oxidation with Ki values of approximately 0.04-3.8 mM for ADH1 and 6-12 mM for ADH4 (28-30). These Ki values are well within the range of blood alcohol concentrations achieved by moderate or binge drinkers (31) and alcoholics have much higher blood ethanol levels (32). Thus, ethanol inhibition of ADH-catalyzed retinol oxidation may be of concern medically as it could result in reduced RA synthesis leading to an inhibition of retinoid signaling, or reduced clearance of retinol leading to toxicity.
Adverse interactions occur when vitamin A and ethanol are administered simultaneously. This is particularly noticeable in alcoholics administered retinol supplements to counter vitamin A deficiency where it is well known that retinol/ethanol drug interactions occur to increase hepatotoxicity and carcinogenicity (33). The mechanism of such interactions is not well understood, but may relate to the fact that ADHs can utilize both retinol and ethanol as substrates. Genetic studies have demonstrated that Adh1−/− mice have large deficiencies in metabolism of both ethanol and retinol, indicating that ADH1 can efficiently metabolize both substrates in vivo (34). In addition to a decrease in metabolism of a dose of retinol, Adh1−/− mice display increased retinol toxicity indicating that clearance of retinol to RA is protective against retinol toxicity (35). These studies have shown that ADH1, expressed at high levels in liver, is the major enzyme responsible for clearance of excess retinol or ethanol. Thus, analogous to the observation that increased retinoid toxicity occurs in vitamin A treated Adh1−/− mice, increased retinoid toxicity associated with alcohol consumption may be rooted in the ability of ethanol to inhibit ADH-catalyzed retinol oxidation.

Here we have examined RA synthesis and degradation in retinoid-treated wild-type mice either with or without additional exposure to acute ethanol intoxication. We have also compared these results with those obtained from Adh1−/− mice. Our results indicate that acute ethanol treatment reduces ADH1-catalyzed metabolism of a dose of retinol resulting in decreased RA synthesis, but on top of this there is also an increase in RA degradation that accounts for some additional loss in RA.

EXPERIMENTAL PROCEDURES

Animals - The wild-type and Adh1−/− null mutant mice used here have been described (34). Both strains are on the same genetic background: 50% C57Bl/6, 25% Black Swiss, 25% 129/SvJ. All mice examined were matched for age, weight, and sex.

Acute Ethanol Treatment - When used, ethanol was administered intraperitoneally as one acute
dose at 3.5 g/kg (18 µl of 25% ethanol in physiological saline per gram body weight) as previously described (34). Control injections consisted of the same volume of physiological saline. Injections were performed 30 min prior to retinol or RA injection.

Retinol and RA Treatment - Retinoids were administered orally essentially as described (36). All-trans-retinol or all-trans-RA (Sigma) were dissolved in acetone-Tween 20-water (0.25:5:4.75 v/v/v) and were administered by oral injection at a dose of 50 mg/kg for retinol or 10 mg/kg for RA. At several time points following retinoid injection, blood was collected and stored at -20°C until HPLC analysis as described below.

Quantitation of Retinoic Acid and Retinol by HPLC - Serum (200 µl) was extracted with 2 ml of methanol-acetone (50:50 v/v). After centrifugation at 10,000 x g for 10 min at 4°C, the organic phase was evaporated under vacuum and the residue was dissolved in 200 µl of methanol-dimethylsulfoxide (50:50 v/v). Samples were analyzed by HPLC to quantitate retinoid levels using standards for all-trans-RA and all-trans-retinol (Sigma). Reversed-phase HPLC analysis was performed using a MICROSORB-MVTM 100 C18 column (4.5 x 250 mm) (Varian) at a flow rate of 1 ml/min. Mobile phase consisted of 0.5 M ammonium acetate-methanol-acetonitrile (25:65:10 v/v) (solvent A) and acetonitrile (solvent B). The A:B (v/v) gradient composition was: 100:0 at the time of injection; 70:30 at 1 min; 65:35 at 14 min; 0:100 at 16 min. UV detection was carried out at 340 nm.

Data Analysis - Quantitation of RA synthesis or degradation over time was performed by determining the area under the curve (AUC). Statistical significance was determined for raw data using the unpaired Student’s t test (Statistica version 5.0).

RESULTS

RA Synthesis During Acute Ethanol Treatment of Wild-Type Mice - In order to examine RA synthesis, mice were treated orally with a single dose of retinol (50 mg/kg) and metabolism to RA was followed over time by quantitation of RA in the serum. RA is normally present in mouse serum
Treatment of wild-type mice with retinol resulted in a peak RA concentration of 1170 ng/ml (Fig. 1A).

We have previously shown that treatment of mice with a single intraperitoneal dose of ethanol (3.5 g/kg) results in a peak blood ethanol concentration of approximately 90 mM at 30 min after the dose, with the concentration dropping to approximately 25 mM at 4 hours post-treatment (34). Mice exposed to acute ethanol intoxication in this fashion were examined for RA synthesis as above.

Pretreatment of wild-type mice with 3.5 g/kg ethanol 30 minutes prior to retinol treatment led to a large reduction in serum RA with the peak RA concentration reaching only 180 ng/ml (Fig. 1). Comparison of area under the curve (AUC) values demonstrated that ethanol pretreatment resulted in an 87% reduction in the level of serum RA (Table I). Whether this is entirely due to a decrease in RA synthesis or whether an increase in RA degradation may also occur during acute ethanol exposure is examined below.

Comparison of RA Synthesis in Wild-Type and Adh1-/- Mice - Treatment of Adh1-/- mice with 50 mg/kg retinol resulted in a peak serum RA concentration of only 270 ng/ml relative to wild-type mice which exhibited 1170 ng/ml (Fig. 1B). AUC values indicated that disruption of Adh1 resulted in an 82% reduction in the level of serum RA following a dose of retinol (Table I). These findings suggest that ADH1 plays a large role in synthesis of RA from the administered retinol, but the effect of a loss of ADH1 on RA degradation is also examined below.

Retinol Clearance - The above mice were also examined for the concentration of serum retinol. Whereas wild-type mice exhibited 1.6 µg/ml serum retinol at 2 hours following retinol treatment, pretreatment with ethanol increased serum retinol to 2.5 µg/ml, and Adh1-/- mice exhibited 3.1 µg/ml (Fig. 2). Significant defects in serum retinol clearance due to ethanol pretreatment or Adh1 disruption were also seen at 4 hours following retinol treatment (Fig. 2).

RA Degradation During Acute Ethanol Treatment of Wild-Type Mice - RA is efficiently metabolized to polar degradation products (38) leading to a half-life in mouse serum of 0.5-1.0 hour (39). Following treatment of wild-type mice with 10 mg/kg RA, serum RA was quantified over time to examine the time-course of clearance (degradation). Treatment with this dose of RA resulted in a peak serum RA concentration of 1550 ng/ml (comparable to the peak RA observed at 4 ng/ml (37).
when retinol was administered above) with almost complete RA clearance observed at 6 hours (Fig. 3A). Pretreatment of wild-type mice with ethanol (3.5 g/kg) 30 minutes prior to RA treatment resulted in a lower RA peak concentration of 1002 ng/ml (Fig. 3A). The AUC values indicated that ethanol increased RA clearance (degradation) by 42% (Table I). Although significant, the effect on degradation is not enough to account for the lower RA values observed in Fig. 1A when retinol was metabolized to RA in the presence of ethanol.

**Comparison of RA Degradation in Wild-Type and Adh1⁻/⁻ Mice** - Treatment of Adh1⁻/⁻ mice with 10 mg/kg RA resulted in a peak serum RA concentration of 1250 ng/ml relative to wild-type mice which exhibited 1550 ng/ml (Fig. 3B). A comparison of AUC values indicated that disruption of Adh1 resulted in only a 26% increase in RA clearance (Table I). Thus, the effect of Adh1 disruption on RA degradation is low, indicating that the results in Fig. 1B are primarily due to a decrease in RA synthesis from retinol.

**DISCUSSION**

The investigation described here indicates that acute alcohol intoxication does in fact reduce metabolism of a dose of retinol and has a negative effect upon RA concentration. A single dose of ethanol was shown to decrease RA synthesis plus increase RA degradation, with the effect on synthesis being larger. As the peak RA concentrations observed following either 50 mg/kg retinol (1170 ng/ml) or 10 mg/kg RA (1550 ng/ml) are in the same order of magnitude, the studies performed here with retinol treatment or RA treatment can be compared to estimate the relative effect of ethanol on RA synthesis as opposed to degradation. From the AUC calculations it is clear that ethanol decreases the amount of RA measured following retinol treatment by 87%, from an AUC value of 2.93 to 0.37, while increasing RA degradation following RA treatment by 42%. Thus, if one allows that 42% of the ethanol-induced loss of RA following retinol treatment may be due to increased RA degradation, this would only reduce the AUC from 2.93 to 1.70, accounting for an AUC loss of 1.23. As we observed an AUC of 0.37, this indicates that an ethanol-induced decrease
in RA synthesis must account for the remaining AUC loss of 1.33. Thus, the effect of acute ethanol on RA synthesis is larger than the effect seen on RA degradation, but both are significant.

Further evidence that ethanol inhibits RA synthesis was obtained by our results indicating that the administered retinol was not cleared as efficiently during acute ethanol intoxication. This suggests that oxidation of retinol to retinal by ADH was reduced by ethanol, thus producing less retinal for synthesis of RA. Our studies on Adh1<sup>-/-</sup> mice have confirmed that this is the most plausible explanation. In the absence of ethanol, Adh1<sup>-/-</sup> mice demonstrated an 82% reduction in RA concentration relative to wild-type mice. This is nearly the same effect that was observed when wild-type mice were treated with ethanol. Examination of the effect of Adh1 disruption on RA degradation indicated that RA degradation was increased by only 26%. Thus, for both Adh1<sup>-/-</sup> mice and acute ethanol-treated mice, the negative effect on RA synthesis is still greater than the positive effect on RA degradation.

It is unclear why a loss of ADH1 or acute ethanol treatment would increase RA degradation, but it is clear why these conditions would decrease RA synthesis. ADH1 is the most abundant ADH in mouse liver (40-42) and it is the most efficient ADH for ethanol metabolism measured either by <i>in vitro</i> comparison of enzyme activities (40) or by <i>in vivo</i> comparison of ethanol clearance capabilities in wild-type and ADH-deficient mice (34). ADH1 is also the most efficient enzyme for metabolism of a dose of retinol to RA, with ADH3 (expressed ubiquitously) playing a lesser role, but still significant (43). Adh1<sup>-/-</sup> mice do not have noticeable developmental defects, but Adh3<sup>-/-</sup> mice have reduced survival and growth that is rescued by retinol supplementation (43). Thus, ADH3 in contrast to ADH1 has been shown to be necessary for metabolism of retinol to RA when retinol levels are low (physiological). However, a large role for ADH1 in clearance of pharmacological doses of retinol to prevent toxicity has been demonstrated; i.e. Adh1<sup>-/-</sup> mice have increased retinol toxicity as demonstrated by a 3-fold decrease in the LD-50 for retinol (35).

As metabolism of retinol by ADH1 is competitively inhibited by ethanol with a Ki of 0.04-3.8 mM (28,29), the mechanism behind the effects we observed during acute ethanol treatment could involve ethanol inhibition of ADH1-catalyzed retinol metabolism. The dose of ethanol administered here (3.5 g/kg) has been previously shown to result in a peak blood ethanol
concentration of 90 mM after 30 min, then falling to 20 mM during the subsequent 4 hours (34). Thus, during the 4 hours when RA was monitored here after such ethanol treatment, there was clearly sufficient ethanol to almost totally inhibit ADH1-catalyzed retinol oxidation. In order to account for the lower AUC in ethanol-treated wild-type mice (0.37) compared to Adh1⁻/⁻ mice (0.52), it is possible that this is due to ethanol inhibition of ADH3 (43) or yet other ADHs (27) that may play minor roles in clearance of excess retinol. Alternatively, the difference may be accounted for by increased RA degradation as we demonstrated that ethanol-treated mice appeared to have a larger increase in RA degradation than Adh1⁻/⁻ mice.

Collectively, the above findings indicate that ADH1 plays a central role in retinol/ethanol drug interactions, thus suggesting that the primary defect leading to adverse interaction may be reduced clearance of retinol to RA through the ADH metabolic pathway. ADH1 metabolism of retinol evidently produces less toxicity than either accumulation of retinol or metabolism through other pathways.

In addition, the observations reported here on acute ethanol intoxication demonstrate that ethanol decreases RA synthesis from the administered retinol, although it remains to be tested whether acute treatment reduces endogenous RA levels in mice not treated with retinol. Previous studies have demonstrated that chronic ethanol intoxication does reduce endogenous RA levels in rats, and the mechanism of RA deficiency is dependent to a large extent upon an ethanol-induced increase of CYP2E1-dependent degradation of RA to less active metabolites such as 4-oxo-RA (22). However, any effect of ethanol on endogenous RA levels during acute ethanol intoxication (i.e. binge drinking) should be different since CYP2E1 induction requires several days to weeks of continuous ethanol treatment (23). Also, as RA levels during chronic ethanol intoxication were restored to only about 75% of control levels when RA degradation was inhibited by co-administering chlormethiazole, a specific CYP2E1 inhibitor (22), it is unknown what is responsible for the remaining 25% reduction in RA concentration during chronic ethanol intoxication. Our findings here combined with previous studies (43) suggest that this may be due to ethanol inhibition of endogenous RA synthesis catalyzed by one or more retinol-utilizing ADHs.
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Table I.

*Area under curve (AUC) values for the effect of either ethanol or Adh1 gene disruption on all-trans-retinoic acid (RA) synthesis or degradation*^a^  

| Genotype treatment | all-trans-retinoic acid AUC (µg x hr/ml) | % change |
|--------------------|-----------------------------------------|----------|
| RA Synthesis:      |                                         |          |
| (1) wild-type Retinol (50 mg/kg) | 2.93 | - |
| (2) wild-type Retinol (50 mg/kg) + ethanol (3.5 g/kg) | 0.37 | -87% |
| (3) Adh1^-/- Retinol (50 mg/kg) | 0.52 | -82% |
| RA Degradation:    |                                         |          |
| (1) wild-type RA (10 mg/kg) | 4.90 | - |
| (2) wild-type RA (10 mg/kg) + ethanol (3.5 g/kg) | 2.84 | -42% |
| (3) Adh1^-/- RA (10 mg/kg) | 3.65 | -26% |

^a^ Values were calculated from data shown in Figs. 1-4 in which retinol or RA was administered orally with or without ethanol pretreatment intraperitoneally 30 min prior to retinoid administration.

^b^ \([\text{AUC (2 or 3) - AUC (1)}] \times 100 \div \text{AUC (1)}\)
FIGURE LEGENDS

Fig. 1.  **Effect of acute ethanol treatment and Adh1−/− genotype on RA synthesis.**  *A*, All-trans-retinoic acid (RA) was quantitated in the serum of wild-type mice treated with 50 mg/kg retinol (WT control) or in wild-type mice that had also been pretreated with 3.5 g/kg ethanol 30 min prior to retinol treatment (WT+ethanol). All values are mean ± s.e (*n*=3).  *B*, All-trans-retinoic acid (RA) was quantitated in the serum of wild-type (WT) mice or *Adh1−/−* mice treated with 50 mg/kg retinol. All values are mean ± s.e (*n*=3). The WT data in *A* and *B* are identical.

Fig. 2.  **Effect of ethanol or *Adh1−/−* genotype on retinol clearance.**  All-trans-retinol was quantitated in serum of wild-type (WT) or *Adh1−/−* mice at 2 h or 4 h after a 50 mg/kg dose of retinol. The (WT+ethanol) group were pretreated with 3.5 g/kg ethanol 30 min prior to retinol treatment. All values are mean ± s.e (*n*=3). *, *P* < 0.05 (significantly different from the WT value).

Fig. 3.  **Effect of acute ethanol treatment and *Adh1−/−* genotype on RA degradation.**  *A*, All-trans-retinoic acid (RA) was quantitated in the serum of wild-type mice treated with 10 mg/kg RA (WT control) or in wild-type mice that had also been pretreated with 3.5 g/kg ethanol 30 min prior to RA treatment (WT+ethanol). All values are mean ± s.e (*n*=3).  *B*, All-trans-retinoic acid (RA) was quantitated in the serum of wild-type (WT) mice or *Adh1−/−* mice treated with 10 mg/kg RA. All values are mean ± s.e (*n*=3). The WT data in *A* and *B* are identical.
Fig. 1
Fig. 2
**Fig. 3**

A.

- WT control
- WT + ethanol

B.

- WT
- Adh1 -/-
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