Metabolic Deficiencies in Alcohol Dehydrogenase Adh1, Adh3, and Adh4 Null Mutant Mice

OVERLAPPING ROLES OF Adh1 AND Adh4 IN ETHANOL CLEARANCE AND METABOLISM OF RETINOL TO RETINOIC ACID*

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Targeting of mouse alcohol dehydrogenase genes Adh1, Adh3, and Adh4 resulted in null mutant mice that all developed and reproduced apparently normally but differed markedly in clearance of ethanol and formaldehyde plus metabolism of retinol to the signaling molecule retinoic acid. Following administration of an intoxicating dose of ethanol, Adh1 −/− mice, and to a lesser extent Adh4 −/− mice, but not Adh3 −/− mice, displayed significant reductions in blood ethanol clearance. Ethanol-induced sleep was significantly longer only in significant reductions in blood ethanol clearance. Ethanol administration was increased 3-fold in reduced LD50 value. Retinoic acid production following doses of ethanol, Adh1 −/− mice, and 1.5-fold in Adh4 −/− mice but was unchanged in Adh3 −/− mice. Formaldehyde toxicity studies revealed that only Adh3 −/− mice had a significantly reduced LD50 value. Retinoic acid production following retinol administration was reduced 4.8-fold in Adh1 −/− mice and 8.5-fold in Adh4 −/− mice. Thus, Adh1 and Adh4 demonstrate overlapping functions in ethanol and retinol metabolism in vivo, whereas Adh3 plays no role with these substrates but instead functions in formaldehyde metabolism. Redundant roles for Adh1 and Adh4 in retinoic acid production may explain the apparent normal development of mutant mice.

The alcohol dehydrogenase (ADH) family consists of numerous enzymes able to catalyze the reversible oxidation of a wide variety of xenobiotic and endogenous alcohols to the corresponding aldehydes (1). Several distinct classes of vertebrate ADH have been described, all of which are cytosolic and zinc-dependent but differ in substrate specificities and gene expression patterns (2, 3). Three forms that are highly conserved in mammals and other vertebrates are class I ADH (ADH1), class III ADH (ADH3), and class IV ADH (ADH4); see Ref. 1 for ADH nomenclature. Biochemical studies indicate that these three ADHs are able to utilize a wide variety of alcohol and aldehyde substrates in vitro ranging from ethanol to formaldehyde to retinol. However, the precise functions of these enzymes are not yet well established. In humans, ADH4 demonstrates higher retinol dehydrogenase activity than ADH1 with ADH1 having higher ethanol dehydrogenase activity than ADH4 and ADH3 having insignificant retinol or ethanol dehydrogenase activity (4–6). Instead, ADH3 has glutathione-dependent formaldehyde dehydrogenase activity, i.e. upon reaction of formaldehyde with glutathione to produce S-hydroxymethylglutathione, ADH3 oxidizes the hydroxymethyl group to a formyl group to produce S-formylglutathione, which is then the substrate for a hydrolase that regenerates glutathione and produces formate (7).

The potential role of ADH1 and ADH4 in retinol metabolism is particularly interesting because this pathway produces retinoic acid, which is a physiological ligand controlling numerous retinoic acid receptor signaling pathways (8). Also, the potential dual roles of ADH1 and ADH4 as ethanol and retinol dehydrogenases have led us to propose that alcohol abuse may lead to ethanol inhibition of ADH-catalyzed retinol oxidation, hence reduced retinoic acid synthesis, and that this may contribute to ethanol damage such as that seen in fetal alcohol syndrome (9, 10). Physiological data are now needed to complement the available biochemical data to effectively address the in vivo functions of these enzymes, particularly the role in retinoic acid synthesis, which is presently a controversial issue (8).

Genes encoding ADH1, ADH3, and Adh4 have been identified in the mouse, i.e. Adh1, Adh3, and Adh4, respectively (11, 12). An involvement of mouse Adh1 in ethanol metabolism has been proposed (13), but no role in retinol metabolism has been shown. We are not aware of any data linking mouse Adh3 to formaldehyde metabolism. A role for Adh4 in retinoic acid synthesis was first proposed when a retinol dehydrogenase isolated from mouse epidermis was found to be identical to Adh4 (14). Additional evidence linking both Adh1 and Adh4 to retinoic acid synthesis consists of gene expression data where these enzymes show localization in numerous adult retinoid-responsive epithelia including the epidermis (15), male reproductive tract (16), and gastrointestinal tract (17) as well as in retinoid target tissues of embryos at stages E8.5-E9.5 (18, 19) and later embryonic stages (20). Also, retinoic acid and Adh1 plus Adh4 expression have been colocalized in the adult and embryonic adrenal gland, which may function as an endocrine source of retinoic acid (21, 22). We have now generated mice carrying targeted null mutations in Adh1, Adh3, and Adh4. Our results provide genetic evidence demonstrating in vivo functions for these genes in the metabolism of ethanol, formaldehyde, and retinol, with significant overlap of Adh1 and Adh4 in ethanol and retinol metabolism.

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The abbreviations used are: ADH, alcohol dehydrogenase; kb, kilobase(s); E, embryonic day; LORR, loss of righting response; Adh1, mouse class I ADH gene; Adh3, mouse class III ADH gene; Adh4, mouse class IV ADH gene; ADH1B, human class I ADH β gene; tk, thymidine kinase.
**Experimental Procedures**

**Construction of Adh-targeting Vectors—**Gene replacement targeting vectors were produced for all three mouse Adh genes. Genomic clones for mouse Adh1 previously have been described (23, 24). We screened a mouse 129/SvJ genomic library (Stratagene, La Jolla, California) using a mouse Adh1 cDNA (25) and isolated a 9-clone container containing the region spanning from exon 4 to the 3′-flanking region. A SacI-EcoR1 DNA fragment of 2.2 kb containing the 3′-flanking region of Adh1 was blunt end cloned into the XhoI sites of plasmid pTK-neo, which lies between the PGK-neo and PGK-tk gene cassettes (26). A 4.2-kb HindIII fragment containing exon 6 was subcloned into pBluescript II KS, excised with XhoI and NotI, and inserted into XhoI-NotI located downstream of PGK-neo to produce the Adh1 gene-targeting vector. A 129/SvJ genomic clone (λ2-2) for mouse Adh3 containing the 5′-flanking region and exons 1–6 has previously been described (27). A 4.2-kb Sall-XbaI fragment containing the 5′-flanking region was liberated from λ2-2 and inserted into the Sall-XbaI sites of pTK-neo located between PGK-neo and PGK-tk. A 3.7-kb XhoI-NotI fragment containing exons 5 and 6 was liberated from λ2-2 and inserted between XhoI-NotI located downstream of PGK-neo to produce the Adh3 gene-targeting vector. The Adh4-targeting vector has been previously described (28).

**Creation of Adh Null Mutant Mice—**Adh1, Adh3, and Adh4 gene-targeting vectors were all linearized with NotI and introduced by electroporation into mouse embryonic stem cells (R1 cells from 129/Sv strain) using standard methodology (29). To enrich the cells incorporating the constructs by homologous recombination, positive selection was with G418, and negative selection was with ganciclovir. Identification of cells carrying Adh deletions was accomplished by Southern blot analysis of isolated G418-resistant cell clones. Each gene was cloned into pBluescript II KS, excised using NotI-HindIII, and subjected to EcoRV digestion for Southern blot analysis. The PGK-neo and PGK-tk gene cassettes (26). A 4.2-kb HindIII fragment containing the 5′-flanking region was liberated from λ2-2 and inserted into the Sall-XbaI sites of pTK-neo located between PGK-neo and PGK-tk. A 3.7-kb XhoI-NotI fragment containing exons 5 and 6 was liberated from λ2-2 and inserted between XhoI-NotI located downstream of PGK-neo to produce the Adh3 gene-targeting vector. The Adh4-targeting vector has been previously described (28).

**Retinol Treatment and Measurement of Retinoic Acid—**All treatments ethanol was administered intraperitoneally as one acute dose at 3.5 g/kg (18 μl of 25% ethanol in physiological saline/μl of body weight). Control injections consisted of 0.2 ml of vehicle. Northern blot analysis of 10 μg of total RNA from mouse tissues using Adh cDNA probes as described previously (19). Polyclonal antibodies against mouse ADH1, ADH3, and ADH4 were used to perform Western blots of mouse tissues containing 20 μg of total protein as reported (17).

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**Ethanol-induced loss of righting response (LORR) was determined as 32(2) by placing animals upside down in a V-shaped trough immediately upon induction of ethanol-induced sleep and measuring the time until the animal could regain its righting response by being able to right itself three times within a 30 s period.**

**For examination of ethanol-induced embryonic resorption, the day of pregnancy and stage of embryonic development were determined by vaginal plug appearance with noon on the day of plug detection being considered E0.5 (34).**

**Formaldehyde Treatment—**All mice treated with formaldehyde for determination of LD₅₀ values were male and were matched for approximate age and weight. Mice were given an intraperitoneal injection of 10% formalin in physiological saline (Sigma) in doses ranging from 0.09–0.22 g/kg (2.5–6.0 μl of 10% formalin/μl of body weight). A lethal dose of formaldehyde was defined as an amount able to result in death within 90 min.

**Retinol Treatment and Measurement of Retinoic Acid—**All trans-retinol (Sigma) was dissolved in a vehicle consisting of acetone/Tween 20/water (0.25:5:4.75) and administered at a dose of 100 μg/kg to adult female mice (age and weight matched) by oral intubation as described previously (35). Retinol solution or vehicle solution (same as above but without retinol added) was administered at 5 μl/g body weight. Two h after administration, tissues were dissected, and retinoic acid was monitored either qualitatively or quantitatively using an F9-RARE-lacZ reporter cell bioassay, which detects the sum of all active carboxylated retinoids including all trans-retinoic acid (21, 36). Qualitative analysis was performed by incubating the tissues as explants on a monolayer of F9-RARE-lacZ reporter cells and examining retinoic acid diffusion by monolayer induction of lacZ expression in the adjacent reporter cells in situ (21). Retinoic acid levels in kidney homogenates were quantitated essentially as described previously (20) by performing a spectrophotometric variation of the reporter cell bioassay with all trans-retinoic acid (Sigma) as the standard; kidney tissue was homogenized for 30 s with a Tissue-Tearor homogenizer (Biospec Products, Inc.) and immediately centrifuged, and then the supernatant was applied to the reporter cells.

**Statistics—**Statistical significance was determined for raw data using Fisher’s exact test (two-sided), two-way analysis of variance (ANOVA), or the unpaired Student’s t test (two-tailed) (GraphPad Prism version 2.0b, GraphPad Software, Inc., San Diego, California).

**Results and Discussion**

**Production of Adh Null Mutant Mice—**Shown are the targeting vectors used to inactivate Adh1 (Fig. 1A) and Adh3 (Fig. 2A) as well as maps of the wild-type and mutant loci; a null mutant for Adh4 has been previously described (28). For each mutation a gene replacement strategy was employed that deletes a portion of the gene, i.e., deletion of exons 7–9 in Adh1 and deletion of exons 1–4 in Adh3. Following introduction of each gene-targeting vector into embryonic stem cells and Southern blot analysis of approximately 100 independently selected clones for each targeting event, we retrieved two clones carrying the expected Adh1 heterozygous mutation identified by a 4.1-kb...
protein in homozygous type mice (Sto stomach (protein from homogenates of epididymis (Epi demonstrates a mutant locus in which exons 1–4 have been deleted.

Expected chimeric males, and at least two males demonstrated germ line into mice. For each were used for blastocyst injection to introduce the mutations

Adh1, Adh3, or Adh4 was not obviously hazardous to prenatal or postnatal survival of mice raised under standard laboratory conditions. Homozygous lines for each Adh mutant were derived, and all further studies were focused upon a comparison of these three –i– lines compared with the +/+ wild-type mice generated during the heterozygous matings.

Effect of Adh1, Adh3, and Adh4 Null Mutations on Ethanol Clearance and Toxicity—As ethanol is often thought to be the main substrate for ADHs, we tested the ability of each null mutant to metabolize ethanol following a large acute dose consisting of 3.5 g of ethanol/kg of body weight. A comparison of blood alcohol clearance in the three Adh mutants and wild-type mice demonstrated that Adh1 –i– mice have a severe defect in clearing ethanol, whereas Adh4 –i– mice demonstrated a noticeable but less severe defect. 360 min after ethanol administration blood ethanol concentrations in Adh1 –i– mice were approximately 0.30% (w/v) with Adh4 –i– mice having levels of about 0.15%, whereas wild-type and Adh3 –i– mice both had levels near the lower limit of detection at 0.03% (Fig. 3). Thus, it can be concluded that Adh3 plays no role in blood ethanol clearance, whereas both Adh1 and Adh4 do with Adh1 playing a much more significant role.

The duration of ethanol-induced sleep following an ethanol dose of 3.5 g/kg was measured by examining the LORR. LORR was significantly longer only in Adh1 –i– mice (85 ± 10 min) as compared with wild-type (45 ± 10 min), Adh3 –i– (45 ± 3 min), or Adh4 –i– (50 ± 5 min) mice, which had similar LORR values (Fig. 4). The significantly increased length of ethanol-induced sleep in Adh1 –i– mice correlates with their greatly decreased blood ethanol clearance relative to the other Adh genotypes. The reduced blood ethanol clearance noticed in Adh4 –i– mice may be responsible for the slight increase in the duration of LORR, but this effect was not statistically significant. The 1.9-fold increase in LORR that we notice in Adh1 –i– mice relative to wild-type mice is similar to the 1.8-fold increase in LORR observed in the Adh1 deficient deer mouse (138 ± 9 min) relative to the wild-type deer mouse (76 ± 4 min); these deficient deer mice also have reduced blood ethanol clearance (32).
We also examined the effect of a 3.5 g/kg ethanol dose given at day E8.5 of gestation on ethanol-induced embryonic resorption at day E12.5. Whereas the rate of resorption in control-treated mice of each genotype was only 5–10%, the rate of resorption in ethanol-treated Adh1 −/− mice rose to 30% (p = 0.003), with ethanol-treated Adh4 −/− mice exhibiting 18% resorption (p = 0.21) and with ethanol-treated Adh3 −/− mice plus wild-type mice having similar resorption rates to control animals (8–10%) (Fig. 5). These results show that the Adh1 −/− genotype correlates more strongly than the Adh4 −/− genotype with ethanol damage and that the Adh3 −/− genotype does not lead to increased ethanol damage.

Previous studies on the genetics of alcohol abuse in humans have shown that polymorphic variants of the human ADH1B gene play a role in ethanol susceptibility (reviewed in Refs. 37 and 38). In particular, the ADH1B*2 allele found commonly in Oriental populations encodes an enzyme with higher activity for ethanol oxidation than the enzyme encoded by the ADH1B*1 allele found commonly in Caucasian populations. A lower incidence of alcoholism is associated with the ADH1B*2 allele, presumably because increased production of acetaldehyde following ethanol consumption leads to alcohol aversion. Our genetic studies in the mouse indicate that a reduction in ethanol clearance because of knockout mutations of ADH1 activity (and to a lesser extent ADH4 activity) will also be important for understanding the mechanism of alcohol abuse. Thus, the persistence of ethanol itself, in addition to its more toxic metabolite acetaldehyde, plays a role in alcohol damage as demonstrated by our studies of ethanol-induced resorption during pregnancy.

Effect of Adh Null Mutations on Formaldehyde Toxicity—As we observed no role for Adh3 in ethanol clearance or toxicity, we examined formaldehyde toxicity because human ADH3 is known to function as a glutathione-dependent formaldehyde dehydrogenase. For Adh3 −/− mice the LD50 for formaldehyde was 0.135 g/kg, significantly less than the LD50 for wild-type (0.200 g/kg) (Fig. 6). The LD50 values for both Adh1 −/− mice (0.175 g/kg) and Adh4 −/− mice (0.190 g/kg) were not significantly different from that of wild-type. Thus, our findings support a role for mammalian ADH3 in the clearance of formaldehyde, a role which has apparently been conserved in most if not all organisms including microorganisms (39), plants (40), and invertebrate animals (41).

Metabolism of Retinol to Retinoic Acid in Adh Null Mutants—Treatment of adult mice with a large dose of retinol has previously been found to result in production of excess retinoic acid (35). We have previously shown that the mouse kidney normally has undetectable levels of retinoic acid using a bioassay (20) and that retinol treatment dramatically increases the level of retinoic acid in wild-type mice but leads to a much smaller increase in Adh4 −/− mice (28). Here, mice of each Adh genotype were treated with retinol (100 μg/kg) or vehicle control, and 2 h later kidney retinoic acid levels were monitored using the bioassay. Whereas vehicle-treated mice of each genotype had undetectable levels of retinoic acid in kidney tissue explants examined using a qualitative version of the bioassay, retinol-treated wild-type and Adh3 −/− mice exhibited comparably high levels of kidney retinoic acid; retinol-treated Adh1 −/− and Adh4 −/− mice had significantly lower levels of kidney retinoic acid than wild-type mice (data not shown). Retinoic acid quantitation was performed on kidney homogenates from treated Adh1 −/−, Adh4 −/−, and wild-type mice using a spectrophotometric variation of the bioassay (20). Kidney retinoic acid levels in all vehicle-treated mice were below the limit of detection (<1 pmol/g). Retinol treatment resulted in kidney retinoic acid levels of 273 ± 186 pmol/g for wild-type mice but only 57 ± 15 pmol/g for Adh1 −/− mice and 34 ± 16 pmol/g for Adh4 −/− mice (Fig. 7). Thus, Adh1 −/− and Adh4 −/− mutations lead to reductions of 4.8- and 8.5-fold, respectively, in the ability to metabolize retinol to retinoic acid. Our finding of a larger role for Adh4 than Adh1 in the metabolism of retinol is
consistent with the higher in vitro catalytic activity of ADH4 as a retinol dehydrogenase relative to ADH1 (4–6).

The above findings provide in vivo evidence that ADH1 and ADH4 can metabolize retinol to retinoic acid when retinol is administered under superphysiological conditions, i.e. a dose that is teratogenic to developing embryos. Thus, these studies do not directly address the ability of ADHs to utilize retinol under conditions of sufficiency to produce the small amount of retinolic acid needed for normal growth and development. However, we have also previously shown that ADH4−/− mice have an increased risk of embryonic lethality during vitamin A starvation, suggesting that ADH4 may facilitate retinol utilization when levels are low (28). Thus, ADH4 is likely to participate in retinol utilization for normal growth and development.

If Adh1 and Adh4 have overlapping roles in the conversion of physiological levels of retinol to retinoic acid, then the apparently normal survival of each mutant when maintained on a standard mouse diet could be explained by one gene compensating for the loss of the other. However, in addition to ADHs, which function as cytosolic retinol dehydrogenases, there exist microsomal retinol dehydrogenases that are members of the short-chain dehydrogenase/reductase enzyme family (42–45). It is possible that one or more members of the short-chain dehydrogenase/reductase family may also function physiologically in the conversion of retinol to retinoic acid, hence allowing Adh1 and Adh4 mutants to grow and reproduce apparently normally. The relative importance of ADHs and short-chain dehydrogenase/reductases may be different under different states of retinol deficiency, sufficiency, or excess.

Production of Adh1/Adh4 homozygous double mutants will serve to further decipher the role of ADHs in physiological retinol utilization, but this will be difficult because these genes have been mapped to the same general region of mouse chromosome 3 (46). We attempted to generate such double mutants by mating Adh1 and Adh4 null mutant mice and looking for crossover events in mice heterozygous for both Adh1 and Adh4 mutations. Examination of approximately 300 individual offspring by Southern blot analysis resulted in no detection of crossover events that would place both mutations on the same chromosome and thus allow generation of double homozygous mutant mice (data not shown). This indicates that the genes are indeed very closely linked, requiring the use of alternative approaches to generate Adh1/Adh4 double mutants.

Conclusions—In summary, genetic studies provide evidence that Adh1, Adh3, and Adh4 null mutant mice have clear defects in ethanol clearance, formaldehyde toxicity, and metabolism of retinol to retinoic acid, respectively. Overlap in function is seen because Adh4 contributes secondarily to ethanol metabolism and Adh1 contributes secondarily to retinol metabolism. Adh3 plays no role in the metabolism of either ethanol or retinol but does play a role in formaldehyde metabolism. The functional overlap noticed between Adh1 and Adh4 is likely to be important both for the utilization of vitamin A and for the mechanism of alcohol abuse. Further genetic studies such as the generation of Adh1/Adh4 double knockouts should provide important information on these topics.

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