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Mitochondrial Aldehyde Dehydrogenase (ALDH2) Polymorphism in AA and ANA Rats: Lack of Genotype and Phenotype Line Differences

TIINA KOIVISTO,* LUCINDA G. CARR,† TING-KAI LI† AND C. J. PETER ERIKSSON*[1]

**Biomedical Research Center, Alko Ltd, POB 350, SF-00101 Helsinki, Finland*
†Departments of Medicine and Pharmacology, Indiana University School of Medicine, Indianapolis, IN 46202-5121

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KOIVISTO, T., L. G. CARR, T.-K. LI AND C. J. P. ERIKSSON. *Mitochondrial aldehyde dehydrogenase (ALDH2) polymorphism in AA and ANA rats: Lack of genotype and phenotype line differences.* PHARMACOL BIOCHEM BEHAV 45(1) 215–220, 1993.—Polymorphism of the gene coding for mitochondrial ALDH2 in humans is known to be associated with differences in alcohol drinking behavior. Recently, two different alleles of the ALDH2 gene, $ALDH2^R$ and $ALDH2^Q$, have been found in rats also and a possible relationship between the frequencies of the two alleles and drinking behavior has been proposed. In this study, we examined whether this polymorphism of ALDH2 was the underlying cause for the previously reported acetaldehyde accumulation in the alcohol-avoiding ANA rat line and, thus, could be one of the factors explaining the differences in alcohol drinking behavior between the ANA and the alcohol-preferring AA rat lines. The experimental animals were genotyped and their mitochondrial ALDH activities and blood acetaldehyde concentrations after ethanol injection were measured. The two lines did not differ in their frequencies of $ALDH2^R$ and $ALDH2^Q$ alleles. Thus, the polymorphism in the ALDH2 gene does not explain the acetaldehyde accumulation in ANA rats and it does not seem to be associated with differences in the alcohol drinking behavior in these rat lines.

Rat ALDH2 polymorphism Acetaldehyde

LIVER aldehyde dehydrogenases (ALDHs) (EC 1.2.1.3 and 1.2.1.5) are enzymes catalyzing the oxidation of a wide variety of aliphatic and aromatic aldehydes, including acetaldehyde, which is formed as an intermediate product during ethanol oxidation. In the rat liver, different ALDH isozymes have been found in the mitochondrial matrix, the mitochondrial outer membrane, microsomes, and the cytosol (19,21,23,30). The specific functions of these different isozymes are still mostly unknown. It is generally accepted, however, that the ALDH isozyme that is located in the mitochondrial matrix and has a low K_m for acetaldehyde (ALDH2) plays the major role in the oxidation of ethanol-derived acetaldehyde (10,26).

In many Oriental populations, 30–50% of the individuals are sensitive to alcohol because of acetaldehyde accumulation after ethanol ingestion (24). The cause for this sensitivity is a variant allele of the ALDH2 gene, $ALDH2^2$, which encodes an enzymatically inactive ALDH2. The variant allele has a single base-pair mutation in the codon for amino acid 487, which is changed from glutamic acid to lysine (32). Several studies have shown that the $ALDH2^2$ allele is rare among alcoholics and thus it seems to protect individuals from alcoholism (14,16–18,25,28).

The possible association between the liver ethanol-metabolizing enzymes and alcohol drinking behavior has also been studied with alcohol-avoiding and alcohol-preferring rodents. The cytosolic ALDHs of alcohol-preferring C57BL/6J and alcohol-avoiding DBA/2J mouse strains have been found to exhibit different kinetic properties (1) and there also seems to be a genetically determined difference in response of hepatic ALDH activity to ethanol exposures between C57BL/6J and alcohol-avoiding BALB/c and 129/ReJ strains (6,31). Further, the activity of ADH-A_2, the main class I alcohol dehydrogenase in the mouse liver, is found to be two times higher in livers of C57BL/6J mice than in DBA/2J mice (5). The most recent finding in this field is the polymorphism of the rat ALDH2 gene (7,8). The ALDH2 enzymes of alcohol-

[1] To whom requests for reprints should be addressed.

preferring (P) and alcohol-nonpreferring (NP) rats were found to show different patterns in isoelectric focusing gels. When the cDNA sequences of these two rat lines were compared, a single amino acid difference was found. Most of the P rats were found to have glutamine (CAG) at position 67 (allele $ALDH2^Q$) whereas most NP rats showed arginine (CGG) at this position (allele $ALDH2^R$). The previously reported cDNA sequence from the Sprague-Dawley rat strain has glutamine at this position (13). Later, the genotypes of a large number of P and NP rats were determined and a significant difference in $ALDH2^Q$ and $ALDH2^R$ allelic frequencies between the lines was found (8).

The present study investigated the possible relationship between frequencies of the variant ALDH2 alleles and the alcohol drinking behavior in another pair of rat lines, the high-drinking AA (Alko, alcohol) and low-drinking ANA (Alko, nonalcohol) lines. These rat lines are derived from a different foundation stock than the P and NP lines and are interesting subjects for this kind of study because remarkable amounts of acetaldehyde are known to accumulate in blood of ANA rats after ethanol injection (9). This acetaldehyde accumulation is possibly a consequence of lower hepatic ALDH activity in the ANA line (9,22). Lower ALDH activity may be one important reason for the lower ethanol consumption of ANA rats as compared with AA rats, but the underlying cause for this difference is still unclear. Thus, we wanted to know whether polymorphism of the ALDH2 gene is associated with acetaldehyde metabolism in AA and ANA rats. To do this, we determined ALDH2 genotypes of a large number of AA and ANA rats and compared these data to isoelectric focusing patterns, mitochondrial ALDH activities, and acetaldehyde levels of the same animals.

METHOD

Animals

AA and ANA rat lines have been developed in the Biomedical Research Center of Alko for high and low voluntary ethanol consumption by selective outbreeding, as described elsewhere (12). Rats used in this experiment were from generation F60. Their age was 3.5–4.5 months and their weight ranged from 200–400 g. They were housed in groups of four to six with free access to standard rat chow (Ewos, Södertälje, Sweden) and tapwater. None of the animals had had previous experience with ethanol.

Measurement of Blood Acetaldehyde

Rats were injected twice IP with 1.5 g/kg ethanol as a 12% (w/v) solution in saline. Tail blood samples were taken 60 and 120 min after injection, hemolyzed, and their ethanol and acetaldehyde contents measured with headspace gas chromatography (11). There was an interval of 1 week between injections. The results are expressed as the means of the two measurements.

Isolation of Rat Liver Mitochondria

Rats were killed by decapitation 4–6 weeks after the second ethanol injection. Livers were removed immediately, and they were made up to 20% with ice-cold sucrose medium containing 0.25 M sucrose, 5 mM Tris, 0.5 mM EDTA, and 2 mM 2-mercaptoethanol (pH 7.2) and homogenized at 800 rpm. The homogenate was centrifuged for 10 min at $1,000 \times g$. The pellet that represented the nuclear fraction was discarded, and the mitochondria were isolated from the supernatant by three centrifugations at $10,000 \times g$ for 10 min following one centrifugation at $20,000 \times g$ for 10 min. The final pellet was resuspended in 20 ml sucrose medium. All steps were carried out at 0–4°C.

Aldehyde Dehydrogenase Activity Assay

Before the aldehyde dehydrogenase activity assay, the samples were solubilized by 0.3% sodium deoxycholate at 4°C for 20 min.

Aldehyde dehydrogenase activity was assayed spectrophotometrically by following the formation of NADH at 340 nm at 25°C. Acetaldehyde was used as the substrate. The assay mixture contained 0.5 mM NAD^+, 0.1 mM 4-methylpyrazole, 2 μM rotenone, and 0.1 or 10 mM acetaldehyde in 60 mM sodium pyrophosphate buffer, pH 8.8. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of NADH per minute in the conditions described above. Enzyme activities are expressed as specific activities (mU/mg protein).

Proteins were assayed using the Bio-Rad Protein Assay (Bio-Rad, Richmond, VA) method according to the instructions of the manufacturer. Bovine serum albumin was used as a standard protein.

Isoelectric Focusing

Ultrathin (0.5 mm) polyacrylamide gels containing 5% acrylamide, 0.2% bis-acrylamide, 10% sucrose, 2% ampho-

TABLE 1

ACETALDEHYDE AND ETHANOL LEVELS IN AA AND ANA RATS

			AcH (μM) (min)*		EtOH (mM) (min)*	
Line	Sex	*n*	60	120	60	120
AA	Male	13	2.3 ± 0.4†	3.7 ± 0.5†	30.9 ± 0.6	21.4 ± 0.6‡
ANA	Male	13	40.4 ± 6.3	39.9 ± 5.8	30.8 ± 0.7	21.5 ± 0.7§
AA	Female	11	3.0 ± 0.4†	3.4 ± 0.9†	32.0 ± 0.8	18.3 ± 0.8
ANA	Female	12	28.2 ± 4.0	28.7 ± 2.8	32.6 ± 0.6	19.1 ± 0.7

Results are expressed as mean ± SEM.

*Blood acetaldehyde and ethanol levels 60 and 120 min after ethanol injection (1.5 g/kg, IP).

Student's *t*-test: †significant ($p < 0.0001$) line differences. ‡§Significant ($p < 0.01$ and $p < 0.05$, respectively) sex differences within the same line.

TABLE 2

HEPATIC MITOCHONDRIAL ALDH ACTIVITIES IN AA AND ANA RATS

Line	Sex	*n*	ALDH 10 mM AcH*	ALDH 0.1 mM AcH*
AA	Male	13	35.5 ± 1.0	6.8 ± 0.7
ANA	Male	13	36.1 ± 1.1†	8.1 ± 0.6†
AA	Female	11	38.0 ± 0.8‡	6.3 ± 0.2§
ANA	Female	12	29.6 ± 1.0	5.1 ± 0.4

Results are expressed as mean ± SEM.

*Specific ALDH activities when 10 and 0.1 mM acetaldehyde are used as substrate. Results are expressed as mU/mg protein.

Student's *t*-test: †Significant ($p < 0.001$) sex differences within the same line. ‡§Significant ($p < 0.001$ and $p < 0.05$, respectively) line differences.

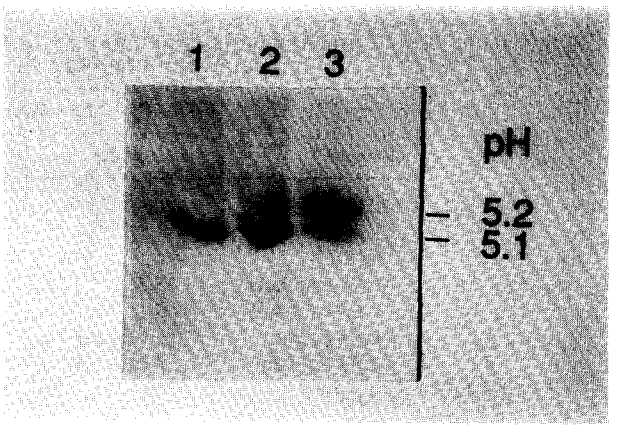

FIG. 1. Isoelectric focusing of ALDH2 from rats with different ALDH2 genotypes. Lane 1, $ALDH2^{Q/Q}$; lane 2, $ALDH2^{R/Q}$; lane 3, $ALDH2^{R/R}$.

lyte (pH range 3.5–9.5), 0.05% TEMED, and 0.04% ammonium persulphate were prepared. Mitochondria samples were treated with 0.2% Triton X-100 and 1 mM dithiothreitol, and they were applied to the gel using sample application foils. The electrode solutions were 25 mM glutamic acid plus 25 mM aspartic acid and 2 M ethylenediamine plus 25 mM lysine plus 25 mM arginine. Isoelectric focusing was carried out at +4°C using an LKB 2117 Multiphor electrophoresis unit. The gel was prerun with 15 mA, 15 W, and max V for 20 min. After application of the samples, the gel was run with 50 mA, 20 W, and max V for 45 min; then, the power was increased to 25 W, and the gel was run for an additional 15 min.

ALDH Activity Staining

The gels were stained for ALDH activity with an agarose overlay technique as described before (15). The *pI*s of the isozymes were determined using LKB pI markers, pI range 4.7–10.6 (LKB, Bromma, Sweden). Blanks were prepared by omitting the substrate.

Genotype Determination

Genotyping was performed using genomic DNA extracted from the spleen. The amplification primers, allele-specific probes, and genotyping method were as described earlier (8).

Statistics

The results are expressed as means ± SEM. The line and sex comparisons of blood acetaldehyde concentrations and liver ALDH activities were performed using Student's *t*-test. The comparisons between genotypes within the same line were performed using a two-way (sex, genotype) analysis of variance.

RESULTS

Blood Acetaldehyde Levels and Mitochondrial ALDH Activities

ANA rats showed significantly higher ($p < 0.0001$) blood acetaldehyde levels after ethanol injection than AA rats (Table 1). The acetaldehyde levels seemed to be somewhat lower in ANA females than in ANA males but because of high standard deviations these sex differences were not significant ($p = 0.124$ for 60-min levels, $p = 0.104$ for 120-min levels).

No significant differences between the lines were observed in the blood ethanol concentrations (Table 1). However, females of both AA and ANA lines had significantly lower ethanol concentrations than males 120 min after ethanol injection ($p = 0.004$ for the AA line, $p = 0.024$ for the ANA line), indicating a sex difference in both lines in the rate of ethanol elimination.

The mitochondrial ALDH activity was assayed using two different acetaldehyde concentrations as substrate (Table 2). With both substrate concentrations, ANA females had significantly lower ALDH activity than AA females ($p = 0.025$ with the lower substrate concentration; $p < 0.0001$ with the higher substrate concentration). There were no significant line differences in males. Significant sex differences ($p < 0.001$) with both lower and higher substrate concentrations were found in the ANA line with female rats displaying lower activities (Table 2).

TABLE 3

ALDH2 GENOTYPE AND ALLELE FREQUENCIES

Line	Genotype Frequency $ALDH2^{R/R}$	$ALDH2^{R/Q}$	$ALDH2^{Q/Q}$	Allele Frequency $ALDH2^{R}$	$ALDH2^{Q}$
AA ($n = 24$)	0.50	0.50	0.00	0.75	0.25
ANA ($n = 25$)	0.56	0.36	0.08	0.74	0.26

TABLE 4

HEPATIC MITOCHONDRIAL ALDH ACTIVITIES AND ACETALDEHYDE LEVELS IN AA AND ANA RATS WITH DIFFERENT ALDH2 GENOTYPES

	AA		ANA	
Genotype	Males	Females	Males	Females
$ALDH2^{R/R}$				
n	7	5	8	6
ALDH				
−10 mM AcH	35.5 ± 1.3	38.1 ± 1.1	37.4 ± 1.4	31.2 ± 1.4
−0.1 mM AcH	6.1 ± 0.7	6.1 ± 0.5	8.8 ± 0.8	5.5 ± 0.7
AcH 60 min	2.1 ± 0.6	3.0 ± 0.7	49.9 ± 5.6	28.1 ± 8.2
AcH 120 min	2.7 ± 0.5	1.3 ± 0.6	47.2 ± 5.7	27.4 ± 5.6
$ALDH2^{R/Q}$				
n	6	6	5	4
ALDH				
−10 mM AcH	35.5 ± 1.7	37.2 ± 1.4	34.0 ± 1.0	29.0 ± 1.0
−0.1 mM AcH	7.5 ± 1.7	6.1 ± 0.3	6.9 ± 0.7	5.4 ± 0.5
AcH 60 min	2.6 ± 0.5	3.1 ± 0.5	26.0 ± 11.0	26.6 ± 2.3
AcH 120 min	5.0 ± 0.6*	5.7 ± 1.2	28.3 ± 10.7	30.9 ± 2.8
$ALDH2^{Q/Q}$				
n	0	0	0	2
ALDH				
−10 mM AcH	–	–	–	25.9 ± 3.0
−0.1 mM AcH	–	–	–	3.5 ± 1.1
AcH 60 min	–	–	–	32.0 ± 2.5
AcH 120 min	–	–	–	30.0 ± 4.0

Results are expressed as means ± SEM. Conditions are as described in Tables 1 and 2.

Two-way ANOVA: *Significant difference between $ALDH2^{R/R}$ and $ALDH2^{R/Q}$ genotypes in the AA line, $F(1, 20) = 15.08$, $p < 0.001$. There was no significant sex difference within the AA line in AcH 120-min concentration, $F(1, 20) = 0.54$, $p > 0.05$.

ALDH Genotypes and Isoelectric Focusing Patterns

Both alleles studied, $ALDH2^{R}$ and $ALDH2^{Q}$, were found in both AA and ANA rat lines. No line difference was observed in the frequencies of the two alleles. In both lines, the $ALDH2^{R}$ allele was more frequent than $ALDH2^{Q}$ (Table 3).

The isoelectric focusing patterns of ALDH2 were determined from all genotyped animals. Three different patterns could be distinguished, and they were found to correspond to the different genotypes (Fig. 1). All animals homozygous to $ALDH2^{R}$ allele (12 AAs and 14 ANAs) showed a predominant cathodic band with *pI* 5.2 and a weaker anodic band with *pI* 5.1. The heterozygous $ALDH2^{R/Q}$ animals (12 AAs and 9 ANAs) showed the same two bands, but the anodic one was clearly predominant. Animals homozygous to $ALDH2^{Q}$ allele (2 ANAs) exhibited a single sharp band with *pI* 5.1.

The possible role of the ALDH2 genotype in acetaldehyde metabolism in AA and ANA rats was studied by comparing the genotyping data with the mitochondrial ALDH activities and blood acetaldehyde levels (Table 4). No significant differences in ALDH activities were found between the different genotypes. In the ANA line, no significant differences in blood acetaldehyde levels were observed with regard to the genotypes either. In the AA line, the acetaldehyde levels 120 min after ethanol injection were significantly higher in animals of the $ALDH2^{R/Q}$ genotype as compared with animals homozygous to $ALDH2^{R}$ allele.

DISCUSSION

The alcohol-preferring P and alcohol-nonpreferring NP rats are found to have different frequencies of two ALDH2 alleles, $ALDH2^{Q}$ and $ALDH2^{R}$, and an association of this polymorphism with alcohol drinking behavior has been proposed (7,8). The present study investigated the possible relationship between frequencies of these ALDH2 alleles and alcohol preference in AA and ANA lines, another pair of rat lines genetically selected for high and low ethanol preference. According to the results of the present study, this polymorphism does not seem to be associated with alcohol preference in these rat lines; the frequencies of the alleles $ALDH2^{Q}$ and $ALDH2^{R}$ were found to be similar in these lines, $ALDH2^{R}$ being more common in both of them. The assumption that the single amino acid difference in enzymes encoded by different ALDH2 alleles would result in different isoelectric focusing patterns was confirmed in this study. Each ALDH2 genotype was found to have a different kind of isoelectric banding. These isoelectric patterns were comparable to those previously reported from P and NP rats (2). A double banding pattern closely resembling the pattern of the $ALDH2^{R/R}$ genotype has also been reported for purified rat ALDH2 enzyme (27). The basic arg-67 residue in $ALDH2^{R}$ is probably responsible for the more cathodic banding of ALDH2 in animals with $ALDH2^{R/R}$ and $ALDH2^{R/Q}$ genotypes when compared with animals homozygous to $ALDH2^{Q}$.

In a previous study, it was suggested that the enzyme encoded by $ALDH2^Q$ (which is more frequent in P rats) could have higher catalytic activity than the enzyme encoded by $ALDH2^R$. This would result in faster acetaldehyde metabolism, which in turn could affect the alcohol drinking behavior (8). In this study, we measured the mitochondrial ALDH activities from the livers of all the genotyped AA and ANA animals, but no significant differences between the genotypes were found. Thus, the single amino acid difference does not seem to drastically alter the catalytic properties of ALDH2 at least with regard to the heterozygotes. Because the frequency of the $ALDH2^Q$ allele was low in both lines and only two animals with the $ALDH2^{Q/Q}$ genotype were found, the possibility still remains open that ALDH activity differences with corresponding acetaldehyde differences could exist if a sufficient number of homozygotes were available for a proper comparison.

Despite the absence of activity differences regarding the different genotypes, indirect evidence for a functional activity difference was observed in the AA line. The acetaldehyde levels at 120 min were significantly lower in animals homozygous to $ALDH2^R$ as compared with heterozygotes suggesting that acetaldehyde elimination was faster in the former. In humans, mildly elevated blood acetaldehyde levels have been related to the positive psychopharmacological effects of alcohol drinking (4). Thus, if the same differences in acetaldehyde elimination rate were found in P rats it could be speculated that the higher frequency of the $ALDH2^Q$ allele in this rat line might be due to these nonaversive effects of acetaldehyde.

Several studies with different rodent lines have shown that brain ALDH activity may correlate more with ethanol preference than does liver ALDH activity (2,3,29). Because ALDH2 exists also in the brain, it is conceivable that the ALDH2 enzymes encoded by different alleles could also have some differences in their function in the brain and, thus, somehow affect ethanol drinking behavior. No significant differences have been found between AA and ANA rats in whole-brain ALDH activity either in homogenates or in subcellular fractions (20). However, some line differences have been observed in specific brain regions and types of brain cells (33).

The ALDH activity of liver homogenate is found to be significantly lower in ANA than in AA rats (Koivisto and Eriksson, manuscript in preparation) but the isozymes responsible for this difference are not known. However, it seems probable that the mitochondrial and especially the microsomal "high K_m" isozymes could be involved [(22); Koivisto and Eriksson, manuscript in preparation]. Whether these ALDH isozymes, and the acetaldehyde metabolism in general, are associated with ethanol aversion of ANA rats will be solved with F_2-generation hybrids from reciprocal crosses of AA and ANA lines.

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