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Biochimica et Biophysica Acta 1762 (2006) 276–283

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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In vivo contribution of Class III alcohol dehydrogenase (ADH3) to alcohol metabolism through activation by cytoplasmic solution hydrophobicity

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Received 8 April 2005; received in revised form 19 November 2005; accepted 21 November 2005
Available online 4 January 2006

Abstract

Alcohol metabolism in vivo cannot be explained solely by the action of the classical alcohol dehydrogenase, Class I ADH (ADH1). Over the past three decades, attempts to identify the metabolizing enzymes responsible for the ADH1-independent pathway have focused on the microsomal ethanol oxidizing system (MEOS) and catalase, but have failed to clarify their roles in systemic alcohol metabolism. In this study, we used Adh3-null mutant mice to demonstrate that Class III ADH (ADH3), a ubiquitous enzyme of ancient origin, contributes to alcohol metabolism in vivo dose-dependently resulting in a diminution of acute alcohol intoxication. Although the ethanol oxidation activity of ADH3 in vitro is low due to its very high K_m, it was found to exhibit a markedly enhanced catalytic efficiency (k_{cat}/K_m) toward ethanol when the solution hydrophobicity of the reaction medium was increased with a hydrophobic substance. Confocal laser scanning microscopy with Nile red as a hydrophobic probe revealed a cytoplasmic solution of mouse liver cells to be much more hydrophobic than the buffer solution used for in vitro experiments. So, the in vivo contribution of high-K_m ADH3 to alcohol metabolism is likely to involve activation in a hydrophobic solution. Thus, the present study demonstrated that ADH3 plays an important role in systemic ethanol metabolism at higher levels of blood ethanol through activation by cytoplasmic solution hydrophobicity.

Keywords: Alcohol metabolism; Class III ADH; Class I ADH; Knockout mouse; Activation by solution hydrophobicity; Acute alcohol intoxication

1. Introduction

Alcohol dehydrogenase (ADH, EC 1.1.1.1) activity in the liver is generally accepted to be the primary factor determining the rate of alcohol metabolism in vivo [1]. However, some aspects of the enzymatic pathways of alcohol metabolism are still controversial, because the existence of another pathway independent of ADH has also been demonstrated [2–4]. The non-ADH pathway, which actually means a pathway independent of Class I ADH (ADH1) [5], is known to be insensitive to pyrazoles (specific inhibitors for ADH) [2] and to have a greater metabolic role at high levels of blood alcohol [3]. Studies on animals genetically lacking ADH1 have revealed that the ADH1-independent pathway accounts for more than 30% of total systemic alcohol metabolism [4,6]. Moreover, this pathway is thought to play a major role in alcohol metabolism in alcoholics, as well as in alcohol metabolism during acute intoxication, because the activity of ADH1 in the liver decreases markedly in both cases [7,8]. The identification of this pathway has long been the subject of a sometimes-heated scientific debate, the main question being whether the microsomal ethanol oxidizing system (MEOS) [2,9–12] or catalase [13–

Abbreviations: ADH, alcohol dehydrogenase; Adh1 or Adh3, mouse Class I or Class III ADH gene; $Adh1^{-/-}$ or $Adh3^{-/-}$, disruption in Adh1 or Adh3; WT, wild type; MEOS, microsomal ethanol oxidizing system; LORR, loss of righting reflex; CNS, central nervous system

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doi:10.1016/j.bbadis.2005.11.008

15] is responsible. MEOS, whose main component is CYP2E1, has been assumed to have a greater role in alcohol metabolism after chronic alcohol consumption due to enzyme induction [9]. However, it has recently been demonstrated that there is no difference in the elimination rate of blood ethanol between CYP2E1-null mice and wild-type mice, even after chronic ethanol feeding [16]. The role of catalase has not been clarified in vivo, either, even though mice genetically lacking catalase have been reported [17]. Furthermore, the first-order kinetics of the elimination of blood ethanol observed at very high concentrations [18,19] suggests the involvement of some kind of very high-K_m enzyme in alcohol metabolism in vivo, since the elimination kinetics cannot be explained by the K_ms of MEOS [9] and catalase [13]. Thus, the enzymatic nature of the ADH1-independent pathway is still unclear.

Mammalian livers are known to contain two ADH isozymes other than ADH1, i.e. ADH2 (Class II) and ADH3 (Class III) [5,20]. ADH2 has little contribution to the liver ethanol activity of mouse, whereas ADH3 accounts for a minor part of the activity with a very high K_m for ethanol [8]. ADH3 is the most evolutionarily constant and ancestral form among all the ADHs hitherto discovered, is present throughout the biological world [21], and is expressed in all mammalian tissues [22]. This housekeeping enzyme has been found to be multifunctional: it plays important roles in formaldehyde metabolism as a glutathione-dependent formaldehyde dehydrogenase [23], in NO metabolism as an *S*-nitrosoglutathione reductase [24], and in the oxidation of retinol for the synthesis of retinoic acid [25].

The role of ADH3 in systemic alcohol metabolism has received little attention due to its very high K_m for ethanol. However, this ADH exhibits the greatest activity toward ethanol at molar concentrations and is the most insensitive to pyrazoles among all classes of ADHs [20]. In addition, its activity toward ethanol at the concentrations found in blood is markedly enhanced by a hydrophobic substance [26,27]. In the livers of alcoholics, the activity of ADH3 increases as the total alcohol intake rises, although that of ADH1 decreases [28]. Recent studies have suggested that ADH3 participates in the first-pass metabolism of ethanol through the stomach [29,30] and in alcohol metabolism in pancreatic acinar cells [31]. These results suggest a possible role of ADH3 in alcohol metabolism in vivo.

In the present study, we investigated the contribution of ADH3 to systemic alcohol metabolism by comparing the pharmacokinetics of blood ethanol in Adh3-null mutant mice to that of wild-type mice. In addition, we examined the apparent discrepancy between our finding of a significant role of ADH3 in ethanol metabolism in vivo and its poor activity toward ethanol in vitro by studying the relationship between its activity and the conditions of the reaction medium.

2. Materials and methods

2.1. Mouse genotypes

Mice carrying homozygous null mutations of Adh1 and Adh3 ($Adh1^{-/-}$ and $Adh3^{-/-}$) [6] were transferred to Nippon Medical School from the Burnham Institute. Wild-type (WT) control mice were generated by mating the C57BL/6 (♀) and 129/Svj (♂) strains (Sankyo Lab, Tokyo) because Adh-null mice are a 50:50 mixture of the two. All of these mice had the same genetic background and were raised on a standard mouse diet (MF pellets, Oriental Yeast Co., Ltd., Tokyo) in a SPF room for experimental animals. Both types of Adh-null mice were apparently normal, except that $Adh3^{-/-}$ mice weighed about 28% less than WT mice, as described previously [25]. All animals received humane care in compliance with our institutional guidelines "The Regulations on Animal Experimentation of the Nippon Medical School", which is based on "The Guidelines of the International Committee on Laboratory Animals 1974".

2.2. In vivo studies

2.2.1. Administration of ethanol to mice

Male mice (8–12 weeks old) were injected intraperitoneally with a 13.4, 20.1, 26.8, 30.2, or 33.5% (w/v) ethanol solution in physiological saline (15 μl/g of body weight), resulting in an ethanol dose of 2.0, 3.0, 4.0, 4.5, or 5.0 g/kg, respectively. Five mice of each genotype were given each dose, except for the 5.0-g/kg dose, for which 10 mice per genotype were used. The injection of ethanol was performed between 9:00 and 11:00 a.m., and the treated mice were subsequently provided only with water during the experiments.

2.2.2. Blood ethanol concentration and β value

Blood (10 μl) was periodically collected from the tail vein of a mouse after the administration of ethanol and was immediately sealed in a vial containing 1 ml of 0.0025% n-propanol in saline solution as an internal standard. Blood ethanol concentration was measured with a head-space gas chromatograph (Perkin-Elmer Head Space Analyzer 8500 with a fused silica capillary column [PEG-20M; 3.0-μm film thickness, 25 m×0.53 mm bore, Quadrex Corp. Conn.]) at an oven temperature of 60 °C and an injection temperature of 150 °C using a flame ionization detector at 200 °C after preincubation in a HS-6 head-space sampler at 65 °C for 30 min. The elimination rate of blood ethanol (β value) was obtained from the slope of a regression line fitted to the pseudo-linear part of a blood ethanol curve by the linear least-squares method [32].

2.2.3. Loss of Righting Reflex (LORR) and mortality due to ethanol administration

Ethanol-induced LORR was assessed by placing mice ($n=5$ for each dose of ethanol) on their backs and measuring the time until the reflex recovered, as described elsewhere [33]. The mortality of mice ($n=10$) was observed for 6 h after ethanol administration at doses of 4.5 and 5.0 g/kg.

2.3. In vitro analyses of ADH

The liver and stomach extracts of untreated mice of each genotype (9 weeks old, ♂) were obtained by centrifugation (105,000×*g* for 1 h at 2 °C) after tissue homogenization in 6 volumes (w/v) of extraction buffer (10 mM Tris–HCl, 0.5 mM NAD, pH 8.0) on ice. The electrophoresis of tissue extract and the activity staining of ADH on the gel were performed as previously described [34]. The ADH activity of liver extract was assayed in 0.1 M Na, K-phosphate buffer (pH 7.4) at 37 °C with various concentrations of ethanol as a substrate, and the production rate of NADH was monitored with a fluorescence spectrophotometer (excitation at 350 nm, emission at 466 nm) (FP-770F, Japan Spectroscopic Co., Tokyo) [27]. The effects of hydrophobic substances on the ethanol oxidation activity of ADH3 were studied using purified ADH3 prepared from WT-mouse liver, as described previously [34]. The protein concentration was determined using Coomassie protein assay reagent (Pierce, Rockford, Ill).

2.4. Evaluation of cytoplasmic solution hydrophobicity of liver cells

The liver from a WT mouse (9 weeks old, ♂) was washed, and sliced into 60-μm-thick sections in PBS on ice. The tissue was mounted on a micro slide glass (Matsunami Glass IND. Co. Ltd., Osaka, Japan) and stained with 10 μl of Nile red (40 μg/ml), which acts as a fluorescent probe of hydrophobicity [35]. The stained preparation was observed using a fluorescence microscope (BX50,

Olympus, Tokyo, Japan) at excitation wavelengths of 400–440 nm and emission wavelengths of 480–700 nm through a dichroic mirror (DM455). For the same sample, the emission fluorescence spectrum of Nile red was measured with a photonic multichannel analyzer (PMA-11; Hamamatsu Photonics, Shizuoka, Japan), installed on the fluorescence microscope [36]. The intensity of the fluorescence spectrum for each specimen was normalized by adjusting the scale of the *y* axis to place the maximum peak height at 1.0 on a full-chart scale. The fluorescence intensity of unstained liver tissue was expressed as the ratio of its peak height to that for stained liver tissue. In order to measure the spectrum for cell membranes, a sample of cell ghosts was prepared by bursting mouse red cells in distilled water and centrifuging them 3 times at 3000 rpm.

To further analyze the Nile red fluorescence of liver cells, the same sample was observed using a confocal laser scanning microscope (CLSM-GB; Olympus, Tokyo, Japan) with an argon laser at an excitation wavelength of 488 nm and a band-pass filter for emission wavelengths above 640 nm. A flame scan was performed with 4× integration at a scanning speed of 512 lines/2 s (1 scan comprised 515 pixels). An image was divided into 256 phases of fluorescence intensity from black to white [36,37].

2.5. Statistics

The elimination curve of blood ethanol, the β value, and the duration of LORR were tested by an analysis of variance (one or two-way ANOVA) for mouse genotype, time, and ethanol dose using the standard statistics software "Stat View, Version 4.5" (Abacus Concepts, Inc., NC). The interaction between factors was also tested by two-way ANOVA. The β values of the two genotypes were compared at each ethanol dose by Student's *t* test. Results were considered statistically significant when $P<0.05$.

3. Results

3.1. Blood ethanol concentration and elimination rate in Adh-null mice

The maximum concentrations of blood ethanol, C_{max}, on an average among the three genotypes of mice, reached about 40, 62, 87 or 91 mM for an ethanol dose of 2, 3, 4 or 4.5 g/kg, respectively. Ethanol blood curves exhibited pseudo-linearity from 1.0 to 3.0 h after ethanol administration for a 2.0 g/kg dose; from 1.0 to 5.0 h for 3.0 g/kg; from 2.0 to 8.0 h for 4.0 g/kg; and from 2.0 to 10 h for 4.5 g/kg. The behavior of blood ethanol concentration over time in Adh3$^{-/-}$ mice was markedly different from that in WT mice for all doses of ethanol tested in the range of 2.0–4.5 g/kg ($P<0.005$–0.0001 by two-way ANOVA). The interaction between mouse genotype and time was also seen at doses of 3.0, 4.0 and 4.5 g/kg ($P<0.005$–0.0001).

Fig. 1 compares the β values for various doses of ethanol for Adh3$^{-/-}$ and WT mice. The dose had a markedly different effect on the β value in the two types of mice. Although the value decreased dose-dependently in both types, it decreased more sharply for Adh3$^{-/-}$ than for WT mice ($P<0.005$ for genotype, $P<0.0001$ for the interaction between genotype and dose); and the value for Adh3$^{-/-}$ mice was significantly lower than that for WT mice at large doses of ethanol ($P<0.005$–0.0001 for doses of 3.0, 4 .0, and 4.5 g/kg by *t* test). The rate of ethanol elimination from the body (mg/kg/h), which is given by the ethanol dose divided by the elimination time of blood ethanol, was also significantly lower for Adh3$^{-/-}$ mice than for WT mice ($P<0.05$ for genotype, $P<0.0001$ for the interaction between genotype and dose).

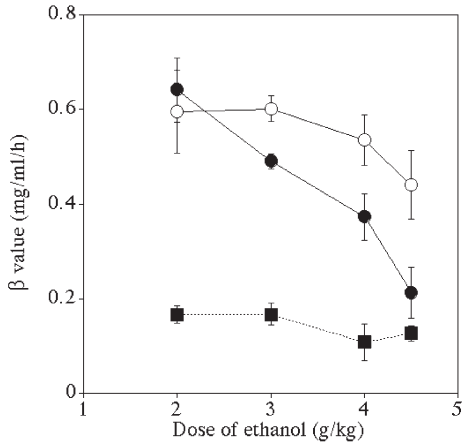

Fig. 1. Comparison of elimination rates of blood ethanol (β values) in Adh3$^{-/-}$ mice (–●–) and WT mice (–○–) for various doses of ethanol ($n=5$ for each dose). The difference in β value curves between Adh3$^{-/-}$ and *WT* mice were significant ($P<0.005$ by ANOVA), as was the interaction between genotype and ethanol dose ($P<0.0001$). –■–: Adh1$^{-/-}$ mice.

As expected, Adh1$^{-/-}$ mice showed a significant difference from WT mice in the behavior of blood ethanol concentration over time and in the interaction between genotype and time at every dose of ethanol ($P<0.0001$). The β value for Adh1$^{-/-}$ mice was the smallest among the three genotypes, and less than one third the value for WT mice at every dose of ethanol (Fig. 1).

3.2. Effect of ethanol on central nervous system of Adh-null mice

We examined the effect of ethanol on the central nervous system (CNS) of the three genotypes by measuring the duration of LORR and mortality. The duration of LORR was significantly longer in Adh3$^{-/-}$ than in WT mice for doses of 4, and 4.5 g/kg (Fig. 2) ($P<0.005$ by two-way ANOVA). The mortality at a dose of 5 g/kg was higher in Adh3$^{-/-}$ than in WT mice (100% vs. 33.3%, $n=10$).

Among the three genotypes, the duration of LORR was the longest for Adh1$^{-/-}$ mice. The time was 5.2 and 6.7 times longer than that of WT mice at doses of 3 and 4 g/kg, respectively. The mortality of Adh1$^{-/-}$ mice was 40% ($n=5$) and 100% ($n=10$) at doses of 4.5 and 5.0 g/kg, respectively.

3.3. ADH isozymes of Adh-null mice

As shown in Fig. 3, the liver of WT mice possessed activity bands for both ADH1 (Class I) and ADH3 (Class III), unlike the livers of Adh3$^{-/-}$ and Adh1$^{-/-}$ mice, which were deficient in the ADH3 and ADH1 bands, respectively. The ADH2 (Class II) band was faintly seen in all three genotypes. The stomach of Adh1$^{-/-}$ mice possessed both ADH3 and ADH4 (Class IV) bands, while that of Adh3$^{-/-}$ mice was deficient in the ADH3 band.

The liver ADH of Adh3$^{-/-}$ mice exhibited lower activities than that of WT mice at ethanol concentrations above 30 mM, indicating the contribution of ADH3 to the liver ethanol

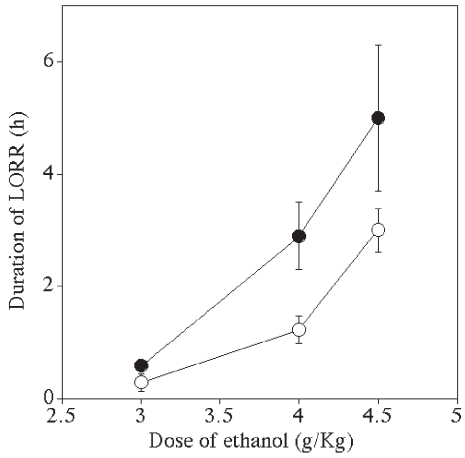

Fig. 2. Effect of Adh3 null mutation on LORR following the administration of various doses of ethanol. The sensitivity was evaluated by measuring the duration of LORR following the administration of ethanol at doses of 3.0, 4.0 and 4.5 g/kg ($n=5$ for each dose). LORR was significantly longer in Adh3$^{-/-}$ (–●–) than in WT mice (–○–) ($P<0.005$ by ANOVA).

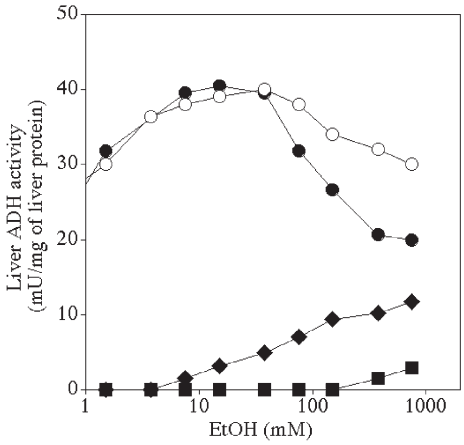

Fig. 4. Liver ADH activities of Adh3$^{-/-}$ (–●–), Adh1$^{-/-}$ (–■–) and WT (–○–) mice at various concentrations of ethanol as a substrate. ADH activity was measured using liver extract at pH 7.4 by monitoring the production of NADH with a fluorescence spectrophotometer. The liver ADH activity of Adh1$^{-/-}$ mice was also measured in the presence of 1.38 M *tert*-butanol as a hydrophobic substance (–◆–).

oxidation activity of WT mice (Fig. 4). Although the liver ADH activity of Adh1$^{-/-}$ mice was very small at the concentrations of ethanol found in the blood due to a deficiency in ADH1, it was greatly enhanced in the presence of the hydrophobic solvent *tert*-butanol (1.38M) (Fig. 4).

3.4. Effects of solution hydrophobicity on ethanol oxidation activity of ADH3

Table 1 shows the effects of hydrophobic substances on the ethanol oxidation kinetics of purified mouse ADH3. Tert-butanol and three kinds of fatty acid amides greatly increased the catalytic efficiency (k_{cat}/K_m) of ADH3 depending on their concentration by markedly reducing K_m. Tert-butanol activated ADH3 the most among the four substances without acting as a substrate for ADH. Fatty acid amides (C4–C6) increased the maximum catalytic efficiency of ADH3, depending on their hydrophobicity.

3.5. Cytoplasmic solution hydrophobicity of liver cells

Fig. 5a shows a fluorescence microscope image of mouse liver tissue stained with Nile red, which acts as a fluorescent probe for hydrophobicity. Lipid droplets (L) in the tissue emitted yellow-gold fluorescence, while liver cells showed a diffuse general staining of the cytoplasm with red-shifted fluorescence. The background (B) of the tissue and the nucleus of the cells (N) were almost black due to the lack of fluorescence. Spectrograms of the emission fluorescence from lipid droplets and liver cells in the same sample are shown in Fig. 5b, together with that from the cell membranes of red-cell

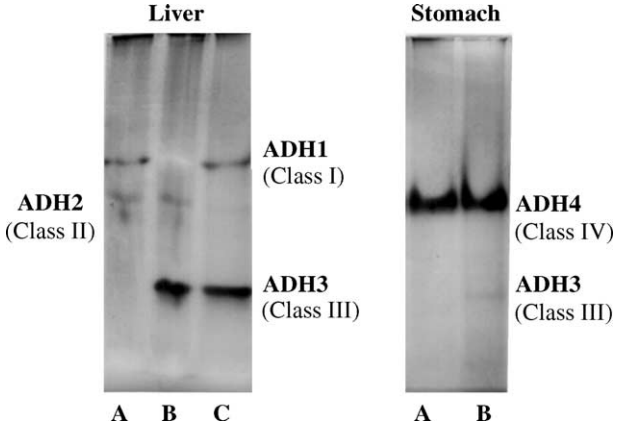

Fig. 3. Zymograms of ADH isozymes in the liver and stomach of Adh3$^{-/-}$, Adh1$^{-/-}$ and WT mice. Tissue extracts were subjected to 7.5% acrylamide gel electrophoresis at pH 9.7. ADH activity was detected by neotetrazolium/PMS staining at pH 10.7, using 7 mM hexenol as a substrate [34]. A, Adh3$^{-/-}$; B, Adh1$^{-/-}$; C, WT mice.

Table 1
Effects of hydrophobic substances on ethanol oxidation kinetics of mouse Class III ADH (ADH3)

Hydrophobic substances	Conc. (M)	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
Non		NS [a]		6.2 [b]
tert-Butanol (C4)	0.12	3.88	37.9	9.8
	0.46	2.24	42.1	18.8
	1.38	0.31	19.4	62.6
Butyramide (C4)	0.10	3.17	26.1	8.2
	1.00	1.66	22.3	13.4
Valeramide (C5)	0.05	2.92	25.1	8.6
	0.50	1.61	24.4	15.2
Capronamide (C6)	0.10	2.38	44.6	18.7
	0.20	0.27	8.6	31.9

Activity of purified ADH3 was measured with ethanol as a substrate in the presence of a hydrophobic substance in 0.1 M Na, K phosphate buffer (pH 7.4) at 37 °C.

[a] NS: Not saturable up to 3 M.

[b] Estimate based on the slope of the curve of velocity vs. substrate concentration $\{v=(k_{cat}/K_m)[E][S]\}$.

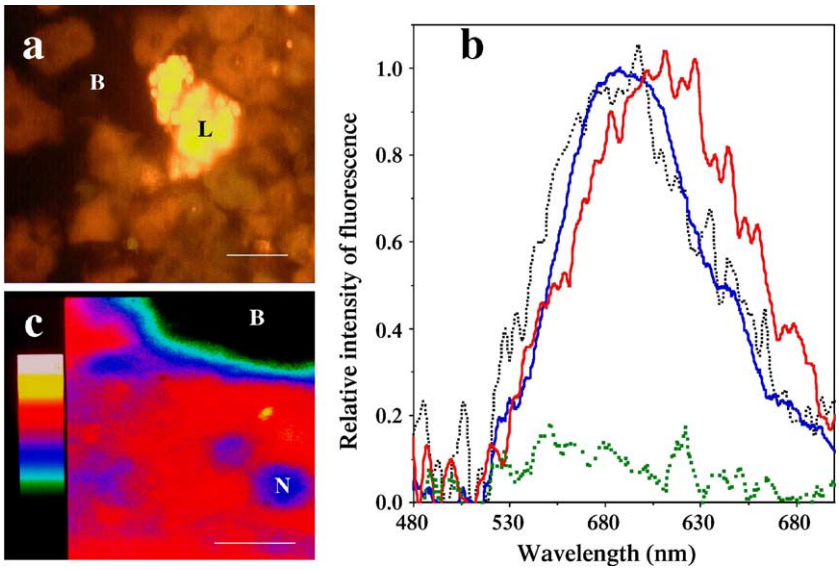

Fig. 5. Evaluation of cytoplasmic solution hydrophobicity of mouse liver by Nile red staining. A liver slice (ca. 60 μm thick) of a WT mouse (9 weeks old) was mounted on a micro slide glass and stained with Nile red as a hydrophobic, fluorescent probe. (a) Nile red fluorescence of mouse liver tissue observed with a fluorescence microscope at excitation wavelengths of 400–440 nm and emission wavelengths of 480–700 nm. B: Background of PBS, L: Lipid droplets. Scale bar, 40 μm. (b) Nile red fluorescence spectra of liver cells (——) and lipid droplets (——) in liver tissue, and cell membranes of mouse red-cell ghosts (----). Unstained liver cells (------). (c) Confocal laser scanning microscope image of Nile red fluorescence of a mouse liver cell. The emission fluorescence was selected with a band pass filter (>640 nm). B: Background of PBS, N: Nucleus of liver cell. Scale bar, 5 μm.

ghosts. The spectrum of liver cells shifted to longer wavelengths (red shift), unlike those of lipid droplets and cell membranes. Fig. 5c shows an image of liver cells observed with a confocal laser scanning microscope (CLSM) with a band-pass filter for wavelengths above 640 nm. As indicated by the red color, the cytoplasm of the liver cell almost uniformly emitted a Nile red fluorescent signal above 640 nm with higher intensities than the background of PBS, indicating that the cytoplasmic solution of liver cells is diffusely hydrophobic. The elevated hydrophobicity of liver cytoplasm relative to non-cellular media suggests that ADH3 ethanol activity is significantly higher in intact cells than in a cell-free assay.

4. Discussion

Although the ADH1-independent pathway is known to play a significant role in systematic ethanol metabolism in mammals, the enzyme responsible for the pathway has not been identified in spite of three decades of effort. However, the present study using Adh3-null mice revealed that ADH3 accounts for the pathway for the state of acute alcohol intoxication, because the β value was significantly lower for Adh3$^{-/-}$ mice than for WT mice at ethanol doses of 3 g/kg and more (Fig. 1). Judging from the data showing that the differences in the β value between the two genotypes increased as the ethanol dose increased (Fig. 1), ADH3 contributes dose-dependently to systemic ethanol metabolism, and keeps the rate of ethanol metabolism constant, irrespective of the ethanol dose. No difference in the β values between the two genotypes observed at a dose of 2 g/kg (Fig. 1) implies that ADH3 has little contribution to systemic ethanol metabolism for the state resulting from ordinary drinking. On the other hand, the contribution of ADH1 to systemic ethanol metabolism decreases dose-dependently, as shown by the marked dose-dependent decrease in the β value of Adh3$^{-/-}$ mice, whose ethanol metabolism depends mostly on ADH1. This is supported by a dose-dependent decrease in the liver ADH1 content [8] and by the substrate inhibition of ADH1 by the formation of dead-end ADH–NADH–ethanol complex at higher concentrations [38]. The substrate inhibition of ADH1 is reflected in the concentration-dependent decrease in the liver ADH activity of Adh3$^{-/-}$ mice, which was observed at ethanol concentrations above 15 mM (Fig. 4).

The effect of first-pass metabolism (FPM) by gastric ADH3, which has been recently suggested by Lee et al. [30], is probably not involved in the results obtained in the this study, because ethanol was administered to mice intraperitoneally (not orally), and at doses of 2 g/kg and more, at which the effect of FPM is not observed on the level of blood ethanol [39].

Thus, ADH3 was demonstrated to play a major metabolic role during acute alcohol intoxication by compensating for the reduced contribution of ADH1. ADH3 may also play an important role in the alcohol metabolism of alcoholics with liver disease, because ADH3 activity in the liver increases as a patient's alcohol intake increases [28], in spite of a decrease not only in the ADH1 activity [7,28] but also in the MEOS [40] and catalase [41] activity in the liver. In addition, ADH3, which is a ubiquitous ADH and the earliest one to appear in vertebrate animals [21], is probably the evolutionary origin of the alcohol-metabolizing enzymes in mammalian cells.

As shown by the LORR data in Fig. 2, the depressant effect of ethanol on the CNS is stronger in Adh3$^{-/-}$ than in WT mice, probably due not only to the delay of systemic ethanol metabolism in Adh3$^{-/-}$ mice, but also to the lack of ADH-dependent ethanol metabolism in the brain because Adh3$^{-/-}$

mice lack ADH3, the sole ADH detectable in the brain [42]. On the other hand, the stronger depressant effect found in Adh1$^{-/-}$ mice (Results) may simply be due to a marked delay of ethanol metabolism (Fig. 1). Thus, not only ADH1 but also ADH3 was found to have a protective role in acute alcohol intoxication.

The finding that ADH3 participates in alcohol metabolism in vivo is seemingly inconsistent with the low level of activity of this ADH toward ethanol in vitro at the concentrations present in blood, which is reflected in the liver ADH activity of Adh1$^{-/-}$ mice (Fig. 4). This seeming discrepancy between in vivo and in vitro results is discussed below.

The low level of ADH3 activity toward ethanol is attributable to the markedly greater volume of its substrate-binding pocket than that of ADH1 due to the substitution of smaller, more hydrophilic residues [43]. However, Moulis et al. [26] and Engeland et al. [44] have suggested that the binding of an anionic hydrophobic ligand (e.g., fatty acid) to Arg-115 of human ADH3 can potentiate the oxidation of ethanol due to structural changes in the binding pocket. We also examined the effects of various hydrophobic substances (19 substances including physiological ones) on the ethanol oxidation activity of purified mouse ADH3 and found that they all enhance the ADH3 activity, regardless of possessing no anion [27] (Table 1). As shown in Table 1, hydrophobic substances with no anion markedly increased the catalytic efficiency (k_{cat}/K_m) of ADH3 in ethanol oxidation due to a marked decrease in K_m. We found that *tert*-butanol activated ADH3 ethanol oxidation almost tenfold without acting as a substrate—the most among various hydrophobic substances (Table 1) [27]. Tert-butanol (C4), which is known to strongly induce solution hydrophobicity in a medium due to its strong hydrophobic hydration and its miscibility, irrespective of the concentration, because of the quadrilateral structure of its molecule [45]. Fatty acid amides also activated ADH3, depending on the lengths of their carbon chain (C4–C6) (Table 1), though their degrees of activation were smaller than those of corresponding fatty acids with an anion (data not shown). These results indicate that the oxidation of ethanol by ADH3 is activated by solution hydrophobicity itself, regardless of what induces it. The liver ADH activity of Adh1$^{-/-}$ mice was also activated by solution hydrophobicity induced by *tert*-butanol (Fig. 4), reflecting an effect on the ethanol oxidation activity of ADH3, an only ADH activated by *tert*-butanol.

We observed no significant changes in the secondary, tertiary or quaternary structures of mouse ADH in hydrophobic solutions (data not shown), therefore, we concluded that solution hydrophobicity induces a slight structural change in the substrate-binding pocket of this ADH3. The hydrophilic amino acids constituting the pocket of ADH3 collapse in a hydrophobic medium, thus reducing the pocket size. Such a structural change in the pocket of ADH3 may raise its affinity for a small molecule like ethanol and thereby increase the ethanol oxidation activity of ADH3. Ebina et al. also [46] reported the effects of hydrophobic substances on the activity of chymotrypsin, and concluded that hydrophobic substances alter hydrophobic interactions within an enzyme protein to change the structure of the active center.

Nile red is strongly fluorescent only in a hydrophobic environment; it fluoresces yellow-gold in lipids and hydrocarbon solvents and does not interact with any tissue constituent except in solution [35]. In an aqueous medium of hydrophobic substances with a polarity such as that of ethanol or phosphatidylcholine vesicles, its fluorescence peak is red-shifted up to 665 nm and quenched, depending on the water content [47]. A cytoplasmic solution of liver cells seems to be in a diffuse hydrophobic state, because the cytoplasm is almost uniformly stained by Nile red and exhibits red-shifted fluorescence, in contrast to the unstained bulk water of PBS (Fig. 5a, c). The diffuse staining of cytoplasm accompanied by the red-shifted fluorescence of Nile red has also been reported for other types of cells [35]. Measurement of the water diffusion coefficient of the cell by NMR also have demonstrated that intracellular water molecules are dynamically restrained by a hydrophobic interaction with various macromolecules and intracellular membranes with an extensive reticulum [48]. Therefore, the in vivo contribution of ADH3 to alcohol metabolism is likely to involve the activation of ADH3 by cytoplasmic solution hydrophobicity, as shown here in vitro (Table 1). Allue et al. [49] reported that, in vitro, the luciferase K_m for ATP in a high-protein milieu shifts markedly to a value similar to that for the enzyme in the cytoplasm of mammalian cells. Thus, some kinds of enzyme activity in a cell could be very different from the activity measured in vitro due to a marked difference in solution conditions between the two milieus. Recent studies on Adh3$^{-/-}$ mice have revealed that ADH3 contributes to systemic retinol metabolism in spite of its extremely low retinol oxidation activity in vitro: it is lower than 0.1% of that observed for ADH1, the key enzyme in systemic retinol metabolism [25].

In conclusion, Adh3-null mice were used to demonstrate that, in spite of its poor ethanol oxidation activity in vitro, ADH3 plays an important role in systemic ethanol metabolism at higher levels of blood ethanol, likely being activated by cytoplasmic solution hydrophobicity, and leads to a diminution of acute ethanol intoxication.

Acknowledgements

The authors would like to thank Dr. Hi. Uedaira of the College of Engineering, Hosei University for his instruction on water science and his critical reading of the manuscript, and Mr. S. Tezuka of the Department of Laboratory Animal Science, Nippon Medical School (NMS) for his assistance in caring for the mice.

This work was financially supported in part by the Japan Society for the Promotion of Science (#11470120).

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