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In vitro inhibition of 10-formyltetrahydrofolate dehydrogenase activity by acetaldehyde*

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Abstract

Alcoholism has been associated with folate deficiency in humans and laboratory animals. Previous study showed that ethanol feeding reduces the dehydrogenase and hydrolase activity of 10-formyltetrahydrofolate dehydrogenase (FDH) in rat liver. Hepatic ethanol metabolism generates acetaldehyde and acetate. The mechanisms by which ethanol and its metabolites produce toxicity within the liver cells are unknown. We purified FDH from rat liver and investigated the effect of ethanol, acetaldehyde and acetate on the enzyme *in vitro*. Hepatic FDH activity was not reduced by ethanol or acetate directly. However, acetaldehyde was observed to reduce the dehydrogenase activity of FDH in a dose- and time-dependent manner with an apparent IC_{50} of 4 mM, while the hydrolase activity of FDH was not affected by acetaldehyde *in vitro*. These results suggest that the inhibition of hepatic FDH dehydrogenase activity induced by acetadehyde may play a role in ethanol toxicity.

Key Words: Ethanol toxicity, acetaldehyde, 10-formyltetrahydrofolate dehydrogenase/ hydrolase, folate

Introduction

Prolonged consumption of excessive amounts of alcohol alters folate metabolism in the liver (Horne *et al.*, 1978; Min *et al.*, 2005; Tamura & Halsted, 1983). Recently, we showed that ethanol feeding decreases 10-formyltetrahydrofolate dehydrogenase (FDH; EC 1.5.1.6.) activity in rat liver and suggested that the inhibition of FDH appears to explain partly a defect of folate metabolism elicited by chronic ethanol ingestion (Im *et al.*, 1998; Min *et al.*, 2005).

FDH is an enzyme that catalyzes the oxidation of formyl group of 10-formyltetrahydrofolate (10-FTHF) to CO_2 in an $NADP^+$-dependent dehydrogenase reaction or an $NADP^+$-independent hydrolase reaction (Kutzbach & Stokstad, 1971). The enzyme is also known as an abundant high affinity folate-binding protein in liver (Cook & Wagner, 1995; Min *et al.*, 1988), a multi-domain enzyme consisting of three domains (Cook *et al.*, 1991; Krupenko *et al.*, 1997), and protein-arginine N-methyltransferase activity (Kim *et al.*, 1998). The $NADP^+$-independent hydrolase reaction occurs in N-terminal domain of FDH, converting to 10-formyl THF to THF and formate, whereas the C-terminal domain is aldehyde dehydrogenase-homologous enzyme capable of $NADP^+$-dependent oxidation (Krupenko *et al.*, 1997). Recently, Donato *et al.* (2008) demonstrated the intermediate domain of FDH as a member of the group of carrier protein with a 4'-phosphopantetheine swinging arm, transferring formyl group between two catalytic domains.

Many of the effects caused by the action of ethanol are actually mediated by its metabolites, namely acetaldehyde and acetate (Israel *et al.*, 1994; Kenyon *et al.*, 1998). Ethanol is metabolized, via three metabolic pathways, to acetaldehyde and to acetate in liver: the cytosolic enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase, the microsomal ethanol oxidizing system (MEOS: cytochrome P450 2E1) and catalase in both peroxisomes and mitochondria (Agarwal, 2001). Generally, acetaldehyde is formed by the action of ADH in liver. However, when ethanol is chronically ingested, cytochrome P450 2E1 (CYP2E1) pathway is induced (Lieber, 1990) and produces much higher concentrations of acetaldehyde (Eriksson & Sippel, 1977). Thus, CYP2E1 induction explains the tolerance to alcohol seen in alcoholics as well (Lieber, 1990).

Since decreased FDH activity *in vivo* by chronic ethanol feeding has been implicated as a toxic effect of ethanol and the action of ethanol may originate from ethanol itself or its metabolites including acetaldehyde and acetate, we investigated the mechanism of this inhibition further *in vitro*. In this study, purified rat liver FDH was used to test *in vitro* effect of ethanol and its metabolites, acetaldehyde and acetate, in time- and dose-dependent studies.

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During this investigation, we found that acetaldehyde inhibited FDH whereas ethanol itself did not inhibit the enzyme.

Materials and Methods

Materials

Sephacryl S-300, DEAE-Sephadex A-50 and Blue Sepharose 6 Fast Flow were obtained from Amersham Biosciences (Piscataway, NJ, USA). Ethanol, acetaldehyde and acetate were purchased from Merck (Whitehouse Station, NJ, USA). Bradford's reagent was obtained from Bio-Rad (Hercules, CA, USA) and [6*RS*]-10-formyltetrahydrofolate was prepared fresh daily from [6*RS*]-5-formyltetrahydrofolate from Sigma (St. Louis, MO, USA). All chemicals were of the highest purity commercially available.

Purification of 10-formyltetrahydrofolate dehydrogenase

Rat livers were removed from Sprague-Dawley rats weighing 250~280 g. FDH was purified from rat liver by the procedure of Scrutton and Beis (1979) with slight modification. Briefly, the liver homogenate was chromatographed on Sephacryl S-300, followed by DEAE-Sephadex A-50active enzyme fractions were then pooled and further purified by Blue Sepharose 6 Fast Flow chromatography. Enzyme with specific activity 0.5 μmol/min/mg protein was used in this study.

Assay of 10-formyltetrahydrofolate dehydrogenase and hydrolase activities

The activity of FDH dehydrogenase was assayed by the procedure of Kutzbach and Stokstad (1971). Assay reaction mixtures (total 2 mL) contained 50 mM Tris-HCl, pH 7.7, 100 mM 2-mercaptoethanol, 100 μM $NADP^+$, 75 μM [6*RS*]-10-FTHF, and aliquots of ethanol, acetate, or acetaldehyde when required, plus the enzyme source. Incubations (30℃) were performed for 3 min and the reaction was terminated by cooling the assay mixture in ice-water bath. The formation of THF was monitored by measuring the increase in A_{300nm} produced by the conversion of 10-FTHF to THF. A millimolar extinction coefficient (corrected for the contribution of NADPH) of 22.6 cm^{-1} was used. Readings were measured against reaction solutions containing no enzyme or no substrate. The activity of FDH hydrolase was assayed by the same methods as FDH dehydrogenase assay in the absence of $NADP^+$.

Protein assay

Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. The absorbance at 280 nm was used to monitor protein in the column effluents.

Inhibition studies for FDH activity by ethanol, acetaldehyde and acetate

Purified enzyme was preincubated in 50 mM Tris-HCl buffer, pH 7.7 in the presence or absence of ethanol, acetaldehyde or acetate to test for enzyme inhibition. Preliminary experiments were carried out to establish optimal conditions. To determine the percentage inhibition, FDH activities from the compound-incubated mixtures were compared to control assays containing equivalent enzyme concentrations in the absence of the compounds. The time-dependent effect of acetaldehyde on FDH activity was analyzed by incubating the purified enzyme in the presence of 2 or 6 mM acetaldehyde at 4℃ for up to 2 hr. The concentrations of acetaldehyde were chosen because free acetaldehyde levels in liver is reported as 250 μM in rats administered ethanol orally (Eriksson & Sippel, 1977) and some acetaldehyde is bound to proteins in liver, so *in vivo* hepatic acetaldehyde concentrations may be higher than these values. Blood acetaldehyde concentrations are higher in the rat than in humans (Kenyon *et al.*, 1998). Since inhibitory activities of acetaldehyde on the dehydrogenase activity of FDH at 4℃ were similar at 37℃ (Fig. 1) and acetaldehyde is volatile, we incubated the enzyme with the agents at 4℃. The hepatic ethanol concentrations were observed as up to 50 mM (Eriksson & Sippel, 1977).

Acetaldehyde solutions were prepared and stored on ice to avoid loss of the material and incubation was performed in light-protected tubes with stoppers. The concentrations of acetaldehyde producing 50% inhibition of the enzyme activity (IC_{50}) was calculated from the least-squares regression line of the logarithmic concentrations plotted the remaining activity.

Results

The dehydrogenase activity of purified rat liver FDH was tested in the presence of ethanol, acetate, or acetaldehyde (Table 1). When FDH was incubated with ethanol for 30 min at 4℃, ethanol had no significant effect on the dehydrogenase activity at concentrations between 10 and 500 mM. However, the dehydrogenase activity was slightly increased in the presence of acetate with concentrations between 10 and 100 mM, possibly reflecting the buffering capabilities of acetate. In contrast to ethanol and acetate, acetaldehyde was found to reduce the dehydrogenase activity of FDH in a concentration dependent way with concentrations between 1 mM and 8 mM acetaldehyde at 4℃ (Table 1). As shown in Fig. 1, acetaldehyde reduced the dehydrogenase activity of FDH at 37℃ similar to at 4℃ (Fig. 1).

The inhibitory effects of acetaldehyde on the dehydrogenase activity of FDH were plotted over 120-min time course in Fig. 2. When FDH was incubated in the presence of 2 mM or 6 mM acetaldehyde at 4℃, acetaldehyde reduced the dehydrogenase activity of FDH immediately and the inhibition developed in full within 120 min in this experimental condition (Fig. 2). The

Table 1. Effects of ethanol, acetate and acetaldehyde on the dehydrogenase activity of FDH *in vitro*

Compounds	Concentration (mM)	% Change in FDH activity[1)]
Ethanol	10	+ 5.4 ± 3.2
	50	+ 9.6 ± 8.8
	100	- 6.5 ± 8.2
	500	+ 8.8 ± 5.9
Acetate	10	+ 2.1 ± 2.4
	50	+ 19.7 ± 7.5
	100	+ 12.1 ± 9.0
Acetaldehyde	1	- 19.8 ± 4.9
	2	- 30.5 ± 5.4
	4	- 50.3 ± 4.2
	6	- 61.1 ± 6.4
	8	- 87.8 ± 10.3

[1)] Purified FDH was preincubated with each compound for 30 min at 4℃. FDH dehydrogenase activity was estimated by THF production at 300 nm in the presence of $NADP^+$ and then by subtracting hydrolytic rate from total rate. Each value represents the mean of triplicate estimates of THF production and expressed as a percentage with respect to a control containing equivalent enzyme concentration and no compound.

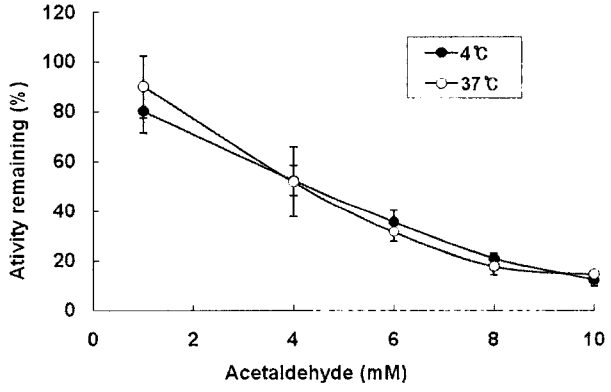

Fig. 1. Effects of temperature on the dehydrogenase activity of FDH during incubation in the presence of acetaldehyde *in vitro*. Purified FDH was incubated in the presence of various concentrations of acetaldehyde at 4℃ and 37℃. FDH dehydrogenase activity was estimated by THF production at 300 nm in the presence of $NADP^+$ and then by subtracting hydrolytic rate from total rate. Each point represents the mean of triplicate estimates of THF production and expressed as a percentage with respect to a control containing equivalent enzyme concentration and no acetaldehyde.

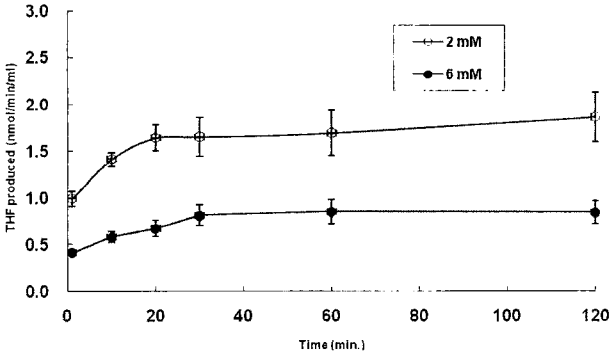

Fig. 2. Effects of acetaldehyde on FDH dehydrogenase activity over time *in vitro*. Purified FDH was incubated in the presence of 2 mM or 6 mM acetaldehyde at 4℃. FDH dehydrogenase activity was estimated by THF production at 300 nm in the presence of $NADP^+$ and then by subtracting hydrolytic rate from total rate. Each point represents the mean of triplicate estimates of THF production.

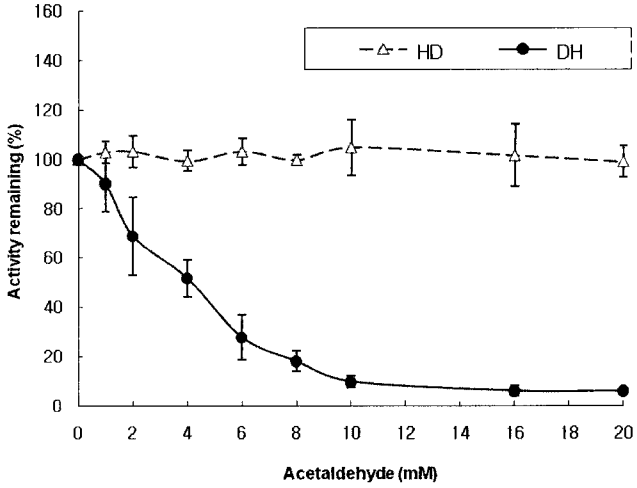

Fig. 3. Effects of acetaldehyde concentration on the dehydrogenase and hydrolase activities of FDH *in vitro*. Purified FDH was incubated in the presence of various concentrations of acetaldehyde at 4℃. Dehydrogenase (DH) activity of FDH was estimated by THF production at 300 nm in the presence of $NADP^+$ and then by subtracting hydrolytic rate from total rate. Hydrolase activity was estimated by THF production at 300 nm in the absence of $NADP^+$. Each point is the mean of triplicate estimates of the activities and expressed as a percentage activity with respect to a control containing equivalent enzyme concentration and no acetaldehyde.

dehydrogenase activities of FDH observed in the presence of 6 mM acetaldehyde were approximately 40 to 60 % lower than the activities observed at 2 mM acetaldehyde during 120 min.

The effect of various concentrations of acetaldehyde on the dehydrogenase and hydrolase activity of FDH was investigated with purified rat liver enzyme. The purified enzyme was incubated with 0 to 20 mM acetaldehyde for 30 min at 4℃ and the enzyme activities were measured (Fig. 3). Acetaldehyde reduced the dehydrogenase activity of FDH with an apparent IC_{50} of 4 mM. When higher concentration of acetaldehyde (≥10 mM) was utilized, the enzyme activity was nearly completely inactivated. In contrast to the inhibitory effect on the dehydrogenase activity of FDH, acetaldehyde had essentially no effect on the rate of hydrolase activity of FDH with concentrations up to 20 mM as determined by production of THF at 300 nm in the absence of $NADP^+$ under standard assay conditions (Fig. 3).

Discussion

Ethanol has long been known to have hepatotoxic effects, even at relatively low doses. Oxidation of ethanol produces acetaldehyde, a highly reactive compound that may contribute to the toxic effect of ethanol in liver (Diehl, 2002; Neuman *et al.*, 2001). The enzymes that are believed to be primarily responsible for the oxidation of ethanol are alcohol dehydrogenase, catalase, and cytochrome P450 2E1 (CYP2E1) (Agarwal, 2001; Oneta *et al.*, 2002).

It has been extensively documented that *in vivo* inhibition of methionine synthase induced by ethanol plays a critical role in

hepatotoxicity and the administration of ethanol depletes hepatic S-adenosylmethionine which protects the liver from ethanol-induced fatty infiltration (Barak *et al.*, 2001; Barak *et al.*, 2003). *In vitro* study with rat liver methionine synthase has demonstrated that inactivation of methionine synthase was induced by acetaldehyde and ethanol did not inhibit methionine synthase directly (*12*) (Kenyon *et al.*, 1998).

We recently observed that chronic ethanol ingestion decreased the hepatic activities of FDH by 46% in rats fed folate-sufficient diets and by 79% in rats fed folate-deficient diets compared to the ethanol-free diet groups (Im *et al.*, 1998; Min *et al.*, 2005). The physiological importance of FDH is established as an abundant folate-binding protein in liver cell (Cook & Wagner, 1995; Min *et al.*, 1988) and a multifunctional enzyme that has three catalytic activities: 1) the $NADP^+$-dependent oxidation of 10-FTHF, 2) the $NADP^+$-independent hydrolysis of 10-FTHF, and 3) the $NADP^+$-dependent oxidation of propanal (Cook *et al.*, 1991; Schirch *et al.*, 1994). In the present study, ethanol, its metabolites acetaldehyde, and acetate were tested to determine their effects on purified rat liver FDH *in vitro*. Our results demonstrated that acetaldehyde reduced the dehydrogenase activity of FDH whereas ethanol and acetate did not induce any significant inhibition.

The ability of acetaldehyde to inhibit FDH dehydrogenase activity was investigated over time-course by incubating FDH with 2 mM or 6 mM acetaldehyde (Fig. 2). The time-dependent experiment revealed that the dehydrogenase activity was inactivated within 1 min, which indicated that the interaction between FDH and acetaldehyde may occur immediately. Dose-dependent study showed that the IC_{50} for the interaction of FDH and acetaldehyde was 4 mM. However, this concentration is not physiologically relevant concentration because free acetaldehyde concentration in rat is reported as 250 nmol/g in liver and 150 μM in the blood (Eriksson & Sippel, 1977). However, the actual concentrations in our *in vitro* system may be much lower than our reported values because acetaldehyde is highly volatile. Furthermore, it is not uncommon to use such concentrations in the *in vitro* studies (Blasiak *et al.*, 2000; Kenyon *et al.*, 1998; Rouach *et al.*, 2005). Thus it may be reasonable to assume that this kind of *in vitro* assay could represent an acute treatment for a short time although the *in vitro* data cannot be directly extrapolated to the *in vivo* situation (Hard *et al.*, 2001). On the other hand, the higher concentrations of acetaldehyde can be achieved by induction of chytochrome P450 2E1 when ethanol is chronically ingested (Agarwal, 2001; Oneta *et al.*, 2002).

We found that hepatic FDH dehydrogenase activity was reduced by acetaldehyde *in vitro* but hydrolase activity was apparently not affected by acetaldehyde *in vitro* (Fig. 3). This observation was different from previous reports in which both dehydrogenase and hydrolase activities of FDH were reduced by chronic ethanol consumption in rats (Im *et al.*, 1998; Min *et al.*, 2005). Since hydrolase reaction of FDH produces THF when hepatic $NADP^+$ levels are low, the hydrolase reaction is proposed as the enzymatic activity used to produce THF irrespective of the redox state of the cell (Rios-Orlandi *et al.*, 1986). However, Krupenko *et al.* (1995) reported that the hydrolase reaction requires millimolar concentrations of ß-mercaptoethanol (ß-ME) *in vitro* while the dependence of the hydrolase reaction on ß-ME is speculated to be unlikely *in vivo*. Therefore, although it is not clear at present why hydrolase reaction is not affected by acetaldehyde *in vitro*, high level of ß-ME in assay media may result in essentially no change in the hydrolase activity in the presence of acetaldehyde in this study.

Although we are unaware of the mechanism how acetaldehyde inhibits FDH, there is increasing evidence that the development of alcohol-related toxicity may involve the formation of protein adducts, which are post-translational modified proteins formed by covalent linkage of acetaldehyde to proteins (Donohue *et al.*, 1983; Worrall & Thiele, 2001). In alcohol toxicity, it was suggested that protein adducts may perturb tissue metabolism by inactivating the affected proteins (Chen *et al.*, 2000), and the affected proteins are more susceptible to proteolysis (Nicholls *et al.*, 1994). Pumford *et al.* (1997) showed that covalent binding of high dose of acetaminophen to FDH was toxic to liver and proposed that the acetaminophen reactive metabolite may bind to sulfhydryl groups of an essential cysteine (cys-707) at the active site of the enzyme. Because FDH has an essential cysteine (cys-707) at the active site (Cook & Wagner, 1995), conversion of cys-707 to alanine in FDH by site-directed mutagenesis led to a complete loss of $NADP^+$-dependent dehydrogenase (Krupenko *et al.*, 1995).

The results of present study show that ethanol does not inhibit FDH directly and acetaldehyde inhibits FDH activity, providing an explanation for the observed inhibition of FDH activity by chronic administration of ethanol to rats. How acetaldehyde decreases the enzyme activity and plays a role in ethanol hepatotoxicity remains to be investigated further.

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