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# THE METABOLISM OF ACETALDEHYDE AND NOT ACETALDEHYDE ITSELF IS RESPONSIBLE FOR *IN VIVO* ETHANOL-INDUCED LIPID PEROXIDATION IN RATS

YOSHIO KERA,* YUMIKO OHBORA and SETSUO KOMURA

Department of Legal Medicine, Kyoto Prefectural University of Medicine, Kamikyo-ku, Kyoto 602, Japan

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**Abstract**—A single oral administration of ethanol (5 g/kg) to rats induced a marked increase in lipid peroxidation, in the liver and kidney within 9 hr, as assessed by malondialdehyde accumulation. The pretreatment with alcohol dehydrogenase (ADH) inhibitor, 4-methylpyrazole (1 mmol/kg) caused approximately 50% inhibition of the hepatic ADH activity and abolished this ethanol-induced lipid peroxidation. The disulfiram treatment (100 mg/kg) significantly inhibited 63% of the hepatic low $K_m$ aldehyde dehydrogenase (ALDH) but not the high $K_m$ ALDH. The cyanamide treatment (15 mg/kg) effectively decreased 83% of the low $K_m$ and 70% of the high $K_m$ ALDH in the liver. Although there was more than a 20-fold elevation of acetaldehyde levels by the inhibition of acetaldehyde metabolism with disulfiram or cyanamide, the ethanol-induced lipid peroxidation was significantly suppressed by pretreatment with these drugs. More than 90% inhibition of xanthine oxidase and dehydrogenase by the pretreatment with allopurinol (100 mg/kg), with no effect on the hepatic ADH and ALDH activities, did not alter the enhancement of lipid peroxidation following ethanol administration.

We propose that the metabolism of acetaldehyde (probably via the low $K_m$ ALDH) and not acetaldehyde itself is responsible for the ethanol-induced lipid peroxidation *in vivo* and that the contribution of xanthine oxidase, as an initiator of lipid peroxidation through acetaldehyde oxidation is minute during acute intoxication.

It has been suggested that lipid peroxidation is one possible mechanism for ethanol-induced liver injury [1, 2]. Despite *in vivo* studies [3–6] in which no sign of the ethanol-induced lipid peroxidation was detected, the enhancement of hepatic lipid peroxidation during the acute ethanol intoxication seems to be well demonstrated by the increase in thiobarbituric acid-reacting substances, mainly malondialdehyde (MAD), with [1, 7, 8] or without [9–11] pre-incubation, diene conjugation in polyunsaturated fatty acids [12–15], chemiluminescence [1, 16] and production of the alkanes [17–19]. Recent *in vitro* [20–22] studies also showed an enhanced production of alkanes induced by alcohols and aldehyde in the isolated perfused rat liver and in the isolated rat hepatocytes. However, the role of ethanol and acetaldehyde metabolism in stimulating lipid peroxidation is not clear, particularly *in vivo*. Microsomes [23, 24], acetaldehyde metabolism via xanthine oxidase [1, 25] and mitochondria from the ethanol treated rat [26] may be responsible for the production of radical species, as an initiator of ethanol-induced lipid peroxidation.

We investigated the role of the metabolism of ethanol and acetaldehyde in increasing lipid peroxidation, using inhibitors for alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and xanthine oxidase. The possible mechanism involved in the initiation of lipid peroxidation is discussed.

* To whom correspondence should be addressed.

## MATERIALS AND METHODS

*Chemicals.* Disulfiram, 4-methylpyrazole (4-MP), allopurinol, diethylmaleate and DL-buthionine-*S,R*-sulfoximine were purchased from Nakarai Chemicals Ltd., Japan. Cyanamide was obtained from Wako Pure Chemicals Industries Ltd., Japan. Thiobarbituric acid (TBA; Nakarai Chemicals) was recrystallized twice with deionized and distilled water. NAD and reduced glutathione were obtained from Sigma Co. (St Louis, MO). A disposable column PD-10 prepacked with Sephadex® G-25M was purchased from Pharmacia (Uppsala, Sweden). Acetaldehyde (Merck Darmstadt, F.R.G.) was redistilled periodically. All other chemicals were of analytical purity.

*Animals and drug administration.* Male Wistar rats weighing 190–220 g were given free access to tap water and a standard laboratory diet and were fasted overnight before experiments.

Ethanol was given by an oral tube in a dose of 5 g/kg as a 25% (w/v) solution, between 8 and 9 a.m. The control rats were given an equal volume of saline. 4-MP was given intraperitoneally (i.p.) in a dose of 1 mmol/kg as a neutralized 0.1 M solution in saline 30 min before the ethanol. Disulfiram was given by an oral tube in a dose of 100 mg/kg as a 2% (w/v) suspension in 5% (w/v) arabic gum 15 hr before the ethanol. Cyanamide was administered i.p. in a dose of 15 mg/kg, in a 0.15% (w/v) solution in saline 1 hr prior to giving the ethanol. Allopurinol was also given i.p. in a dose of 100 mg/kg, in a 1%

(w/v) suspension in saline 1 hr before the ethanol. The control rats were given an equal volume of the vehicle.

*Biochemical determinations.* The animals were decapitated, and pieces of the liver and kidney were rapidly frozen and kept in liquid nitrogen until use (within 3 hr). The frozen tissue was homogenized in cold 154 mM KCl to make up a 10% (w/v) homogenate. The homogenate was filtered through nylon cloth (100 mesh) to remove fragments of connective fibers.

Tissue peroxidation was assessed by measuring the accumulation of TBA reactive substance in the fresh homogenate, without pre-incubation, according to Uchiyama and Mihara [27], but with minor modifications. A mixture including 0.5 ml of the homogenate, 1 ml of 0.67% (w/v) TBA solution and 3 ml of 1% (v/v) $H_3PO_4$ was incubated for 45 min in a boiling water bath. After cooling in an ice-water bath and extraction with 8 ml of *n*-butanol, absorbance of the butanol phase (separated by centrifugation) was measured at 535 and 520 nm. In a preliminary study, addition of acetaldehyde to the homogenate at the final concentrations of 50–1000 $\mu$M had no effect on the absorbance in this assay system. Since there was no difference in the level of hepatic TBA value between the control and the glutathione depleted rats obtained by an acute treatment with diethylmaleate or buthionine sulfoximine, it was suggested that the difference in the endogenous concentration of glutathione did not affect the TBA value in the present assay system (unpublished data). 1,1,3,3-Tetraethoxypropane was used for the standard calculation of TBA reactive substance as malondialdehyde (MAD).

For enzyme assays, rats were decapitated, and liver was perfused with cold saline for 30 sec and frozen in liquid nitrogen. The frozen liver was homogenized in four volumes of cold 50 mM potassium phosphate buffer (pH 7.8). Two and a half ml of a supernatant obtained after centrifugations of 20,000 *g* for 20 min and 105,000 *g* for 60 min was applied on the Sephadex G-25M (PD-10) column, to remove endogenous substrates for xanthine oxidase and dehydrogenase. The samples eluted with 3.5 ml of the homogenizing buffer were analyzed for xanthine oxidase and dehydrogenase activities, by measuring uric acid formation from xanthine at 30° [28]. Protein recovery through the column, using the above method, was greater than 95% (see PD-10 Instructions by Pharmacia). Assay medium for xanthine oxidase contained 100 mM Tris, 0.13 mM sodium EDTA and 60 $\mu$M xanthine, pH 8.1. When the combined oxidase–dehydrogenase activity was determined, 0.6 mM NAD was included [25]. The activity of xanthine dehydrogenase was obtained by subtracting xanthine oxidase from the combined activity. The activity was expressed as nmol uric acid/min per mg protein of the eluted sample.

A 10% (w/v) homogenate was also obtained from the frozen liver with 20 mM sodium phosphate buffer pH 7.4 containing 1 mM reduced glutathione and 1% (v/v) Triton X-100. A supernatant (20,000 *g* for 30 min) was used for assays of ADH and ALDH activities, with 50 mM sodium pyrophosphate buffer, pH 9.0, at 30° [29]. In the ALDH assay, two different concentrations of acetaldehyde (50 $\mu$M for low $K_m$ isozyme or 20 mM for the combined low $K_m$—high $K_m$ isozyme activity) were used as substrate. The activity of high $K_m$ enzyme was calculated by subtracting low $K_m$ enzyme activity from the combined activity. The enzyme activity was expressed as nmol NADH/min per mg protein of the supernatant.

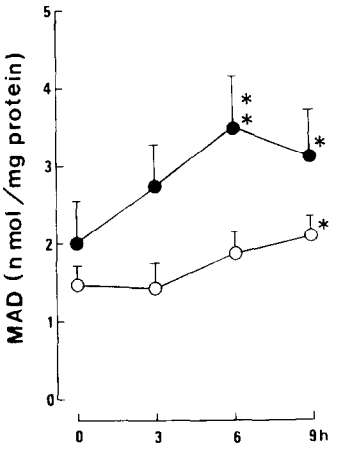


Fig. 1. Accumulation of MAD induced by a single oral administration of ethanol. Each group included four to six rats. ●—●, liver; ○—○, kidney. *P < 0.05, **P < 0.02, as compared to the initial value.

Blood samples were collected from the tip of the tail directly into heparinized glass micropipettes (Clay Adams, U.S.A.) before decapitation, and 8% (v/v) haemolysate with double distilled water was used for the determination of ethanol and acetaldehyde by the head-space gas chromatography [30].

Protein concentration was determined according to Lowry *et al.* [31]. Data are expressed as means ± SD and the statistical significance was determined using Student's *t*-test for unpaired comparison.

## RESULTS

A single oral administration of ethanol (5 g/kg) to rats induced a progressive accumulation of MAD in the liver (Fig. 1), and the level reached a plateau 6–9 hr later. In the kidney, MAD accumulated to some extent at 6 hr and significantly so at 9 hr after the ethanol administration.

Pretreatment with an ADH inhibitor, 4-MP, strongly reduced the hepatic accumulation of MAD to the control level, at 6 hr after the ethanol administration (Table 1). The moderate increase in the renal MAD level was also reduced. The activity of ADH in the liver from the pretreated animal was about 43% of that in the control rat (Table 2), and there was no influence of the 4-MP treatment on other enzyme activities so far examined. The significant inhibition of ethanol metabolism was also suggested by the significantly higher level of ethanol in the blood and by the significantly lower level of acetaldehyde in the pretreated animals (Table 1).

Since it was conceivable that a decreased level of acetaldehyde *in vivo* (Table 1), as induced by

Table 1. Effect of 4-MP on ethanol-induced MAD accumulation in liver and kidney, and on blood concentration of ethanol and acetaldehyde

| | | MAD (nmol/mg prot.) | | Blood concentration | |
|---|---|---|---|---|---|
| | (N) | Liver | Kidney | Ethanol (mM) | Acetaldehyde ($\mu$M) |
| Control | (6) | 2.04 ± 1.06 | 1.58 ± 0.30 | — | — |
| Ethanol | (6) | 3.48 ± 0.92* | 1.74 ± 0.32 | 89.6 ± 8.2 | 10.1 ± 4.5 |
| 4-MP | (6) | 2.12 ± 0.56 | 1.64 ± 0.28 | — | — |
| 4-MP + ethanol | (6) | 1.95 ± 0.26† | 1.59 ± 0.19 | 121.0 ± 8.3† | <2† |

Rats were killed 6 hr after ethanol administration.
* Statistically significant ($P < 0.05$) as compared to control group.
† Statistically significant ($P < 0.02$) as compared to rats given ethanol.

inhibition of the ethanol metabolism might be responsible for the reduced concentration of MAD, a study using the ALDH inhibitors, disulfiram or cyanamide, was carried out to obtain evidence for the continuous presence of higher acetaldehyde concentrations *in vivo*. Although the treatment with these inhibitors slightly decreased the hepatic activity of ADH, the treatment with disulfiram or cyanamide respectively inhibited 63% or 83% of the hepatic low $K_m$ ALDH activity (Table 2). The hepatic high $K_m$ ALDH activity was significantly inhibited only by the cyanamide treatment. These drugs have no apparent effects on the hepatic activities of xanthine oxidase and dehydrogenase. Due to the significant inhibition of acetaldehyde metabolism, an extremely high blood concentration of acetaldehyde was observed in case of pretreatment with disulfiram (20-fold increase) or cyanamide (260-fold) (Table 3). Although treatment with disulfiram in itself seemed to cause accumulation of MAD in the liver ($P < 0.05$), the ethanol-induced accumulation was completely blocked by this treatment (Table 3, A). The moderate accumulation seen in the kidney was also significantly reduced. Pretreatment with cyanamide significantly inhibited the ethanol-induced increase in MAD (Table 3, B).

Pretreatment with the xanthine oxidase inhibitor, allopurinol, caused more than a 90% inhibition of xanthine oxidase and dehydrogenase in the liver, with no significant effect on the ADH and ALDH activities (Table 2). The endogenous level of the hepatic MAD was slightly reduced in the animals pretreated with this drug. However, we observed no effect of this drug on the ethanol-induced accumulation of MAD in the liver and kidney or on the blood concentration of ethanol and acetaldehyde (Table 4).

## DISCUSSION

We obtained evidence that a single administration of a relatively large dose of ethanol induced a significant accumulation of MAD in the liver and kidney of rats, within 9 hr. As noted in our previous study [10], the degree of MAD accumulation by ethanol is much larger in the liver than in kidney. We [10] and Videla *et al.* [8] noted that a lower dose (2 g/kg) did not stimulate the MAD accumulation in the liver. In case of the dose of 2 g/kg, ethanol was metabolized within 5 hr (data not shown), but the blood concentration of ethanol was still high 6 hr after the large dose of ethanol (Tables 1, 3 and 4). It is conceivable that the continuous presence of a high metabolic pressure for a long period may be required to stimulate a substantial accumulation of MAD.

The accumulation of MAD caused by the ethanol administration (5 g/kg) was completely suppressed when animals were pretreated with 4-MP (Table 1), as demonstrated *in vivo* [1] and *in vitro* [32]. The reduced rate of conversion of ethanol to acetaldehyde with 4-MP resulted in a significantly low concentration of blood acetaldehyde (Table 1), an event which might secondarily diminish the metabolic stress of acetaldehyde.

Considering the tissue distribution of acetaldehyde after ethanol administration [29, 33], the presence of

Table 2. Effect of 4-MP, disulfiram, cyanamide and allopurinol on hepatic activities of ADH, low and high $K_m$ ALDH, xanthine oxidase and xanthine dehydrogenase

| | | | ALDH | | Xanthine | Xanthine |
|---|---|---|---|---|---|---|
| | (N) | ADH | Low $K_m$ | High $K_m$ | oxidase | dehydrogenase |
| Control | (4) | 13.3 ± 2.9 | 8.1 ± 0.2 | 20.4 ± 3.3 | 0.33 ± 0.08 | 1.85 ± 0.31 |
| 4-MP | (5) | 5.7 ± 1.0* | 8.3 ± 0.8 | 21.2 ± 2.8 | 0.37 ± 0.07 | 1.95 ± 0.21 |
| Disulfiram | (4) | 11.9 ± 1.3 | 3.0 ± 0.7* | 20.7 ± 1.4 | 0.36 ± 0.08 | 1.92 ± 0.37 |
| Cyanamide | (5) | 11.8 ± 1.9 | 1.4 ± 0.5* | 6.0 ± 0.2* | 0.38 ± 0.09 | 1.94 ± 0.22 |
| Allopurinol | (5) | 14.9 ± 2.7 | 8.2 ± 0.7 | 21.2 ± 2.5 | 0.03 ± 0.03* | 0.17 ± 0.03* |

Rats were killed at the same time interval after drug administration, as in Tables 1, 3 and 4. Enzyme activity measured at 30° was expressed as nmol/min per mg protein.
* Statistical significance ($P < 0.001$) as compared to control rats given saline.

Table 3. Effect of ALDH inhibitors on ethanol-induced MAD accumulation in liver and kidney, and on blood concentration of ethanol and acetaldehyde

| | (N) | MAD (nmol/mg prot.) | | Blood concentration | |
|---|---|---|---|---|---|
| | | Liver | Kidney | Ethanol (mM) | Acetaldehyde ($\mu$M) |
| (A) | | | | | |
| Control | (6) | 1.76 ± 0.48 | 1.64 ± 0.26 | — | — |
| Ethanol | (6) | 3.87 ± 0.46* | 1.85 ± 0.30 | 90.2 ± 18.2 | 9.4 ± 4.7 |
| Disulfiram | (6) | 2.94 ± 1.13 | 1.53 ± 0.30 | — | — |
| Disulfiram + ethanol | (7) | 2.63 ± 0.53‡ | 1.57 ± 0.14† | 87.4 ± 17.3 | 190.5 ± 99.0§ |
| (B) | | | | | |
| Control | (5) | 2.90 ± 0.92 | — | — | — |
| Ethanol | (5) | 4.35 ± 0.29* | — | 83.4 ± 11.0 | 9.7 ± 3.7 |
| Cyanamide | (5) | 2.63 ± 0.41 | — | — | — |
| Cyanamide + ethanol | (5) | 2.96 ± 0.88‡ | — | 98.5 ± 7.4† | 2578.6 ± 319.0§ |

Rats were killed 6 hr after ethanol administration.
* Statistically significant ($P < 0.01$) as compared to control group.
†, ‡, § Statistically significant ($P < 0.05$, $P < 0.02$, $P < 0.001$) as compared to rats given ethanol.

a much higher concentration of acetaldehyde in the liver and kidney than in the blood was expected in the rats pretreated with disulfiram and cyanamide. Nevertheless, the MAD accumulation after ethanol was significantly suppressed (Table 3). This result indicates that it is not acetaldehyde itself but rather the metabolism of acetaldehyde which is responsible for the *in vivo* accumulation of MAD during ethanol intoxication. This conclusion argues against a study [1], in which the treatment with disulfiram prior to ethanol enhanced MAD production and chemiluminescence. However, the enhanced effect was observed only after pre-incubation of the homogenates in air. It is probable that pre-incubation of the homogenate, particularly in air and prior to the assay does not reflect the tissue level of lipid peroxidation, as discussed by Mihara *et al.* [34]. In our study, the MAD level was measured in the fresh homogenate, without pre-incubation. Furthermore, recent *in vitro* studies [20–22] on a perfused liver system led to a similar conclusion that the metabolism of aldehyde derived from ethanol or monoamines is the important event for increased release of ethane or pentane, another marker for lipid peroxidation.

The cyanamide treatment significantly decreased the hepatic low and high $K_m$ ALDH activities, and the disulfiram, on the other hand, inhibited only low $K_m$ ALDH (Table 2). These differences in the inhibitory effect on ALDH isozymes have been noted by Loomis and Brien [35] and Jensen and Faiman [36]. Since both inhibitors significantly suppressed the ethanol-induced MAD accumulation, it is suggested that the acetaldehyde metabolism via low $K_m$ ALDH is probably the most important for the ethanol-induced MAD accumulation *in vivo*.

The production of an active radical species is required for the initiation of lipid peroxidation. A participation of radical species produced in microsomes to ethanol oxidation has been suggested [23, 24]. In addition to the inhibition of ethanol oxidation via ADH, an inhibitory effect of 4-MP on the microsomal oxidation of ethanol [37] may be partly involved in suppression by this drug of the ethanol-induced MAD accumulation, through the reduced production of acetaldehyde. An increased production of radical species is evident only in the microsomes obtained from the chronically treated rats [38–40], and the major part of ethanol oxidation is catalyzed through ADH pathway [33]. Therefore,

Table 4. Effect of allopurinol on ethanol-induced MAD accumulation in liver and kidney, and on blood concentration of ethanol and acetaldehyde

| | (N) | MAD (nmol/mg prot.) | | Blood concentration | |
|---|---|---|---|---|---|
| | | Liver | Kidney | Ethanol (mM) | Acetaldehyde ($\mu$M) |
| Control | (5) | 2.55 ± 1.05 | 1.57 ± 0.16 | — | — |
| Ethanol | (5) | 4.82 ± 0.95* | 1.76 ± 0.23 | 97.1 ± 17.5 | 12.6 ± 4.5 |
| Allopurinol | (5) | 1.84 ± 0.34 | 1.56 ± 0.18 | — | — |
| Allopurinol + ethanol | (5) | 4.54 ± 0.94† | 1.69 ± 0.17 | 107.1 ± 7.4 | 13.9 ± 1.1 |

Rats were killed 6 hr after ethanol administration.
* Statistical significance ($P < 0.005$) as compared to control group.
† Statistical significance ($P < 0.001$) as compared to rats given allopurinol.

the possible contribution of radical species produced in microsomes to the ethanol-induced lipid peroxidation may be a minor fraction during the acute intoxication.

Xanthine oxidase generates $O_2^-$ through the oxidation of acetaldehyde [1, 25]. The $K_m$ value to acetaldehyde, however, is high (>1 mM) [33], compared to the acetaldehyde concentration *in vivo* (Ref. 33 and Tables 1, 3 and 4), during the ethanol intoxication. Furthermore, more than 90% inhibition of xanthine oxidase by pretreatment with allopurinol (Table 2) did not prevent the accumulation of MAD after ethanol (Table 4), and the MAD accumulation was not enhanced even with an extremely elevated concentration of acetaldehyde in rats treated with ALDH inhibitors (Table 3). Therefore, the possible contribution of this enzyme to hepatic and renal lipid peroxidation following ethanol administration is considerably small, *in vivo*.

A significant increase in $O_2^-$ production was noted after 2 and 16 hr in hepatic submitochondrial membranes obtained from rats treated with a moderate dose of ethanol [26]. About 20% of the $O_2^-$ produced by the mitochondria may escape mitochondrial superoxide dismutase and exert actions outside the mitochondria [41]. The inhibition of only low $K_m$ ALDH by disulfiram (Table 2) seems to be adequate to suppress the ethanol-induced MAD accumulation (Table 3). Considering that most of the acetaldehyde derived from ethanol is metabolized by low $K_m$ ALDH [33], most of which is located in the mitochondria [33, 35], it is probable that the continuous stress during acetaldehyde metabolism may cause alterations in mitochondrial functions.

In conclusion, the present results suggest that it is not acetaldehyde itself but rather the metabolism of acetaldehyde which is an important factor related to the ethanol-induced MAD accumulation (i.e. lipid peroxidation) *in vivo*.

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