Effect of chronic ethanol consumption on microsomal lipid peroxidation

Role of iron and comparison between controls

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Microsomes isolated from chronic ethanol-fed rats displayed elevated rates of malondialdehyde production when compared to pair-fed controls, but lower rates when compared to chow-fed controls. These differences did not correlate with total content of cytochrome P-450 or activity of NADPH-cytochrome *c* reductase. Titration curves with the potent iron-chelating agent desferrioxamine revealed that the content of iron was greater in microsomes from the chow-fed and lowest in microsomes from the pair-fed control. However, other variables must also exist since even when excess iron was added to the microsomes, the order of malondialdehyde production remained chow-fed > chronic ethanol > pair-fed control. The variabilities associated with the different controls and the role and content of transition metals such as iron probably contribute towards the divergent effects of ethanol on lipid peroxidation.

Lipid peroxidation *Iron* *Ethanol* *Microsome*

1. INTRODUCTION

Microsomes isolated from chronic ethanol-treated rats have been shown to display increased rates of generation of superoxide anion radical [1], H_2O_2 [2,3], and in the presence of chelated iron, of hydroxyl radical [4,5]. In the presence of metals such as iron, systems producing superoxide and H_2O_2 have been shown to undergo lipid peroxidation [6,7]. Hence, it is interesting to speculate that increased generation of toxic active oxygen intermediates could result in enhanced rates of lipid peroxidation. The role of lipid peroxidation in the development of ethanol-induced hepatotoxicity is controversial with some investigators reporting an increase in diene conjugation or thiobarbituric acid-reactive metabolites (malondialdehyde) after ethanol administration [8–11] whereas others found little or no effect by ethanol [12–14]. Since lipid peroxidation is a complex process many of the above differences in the effects of ethanol could reflect the variable reaction conditions utilized, e.g. sex, size, nutritional status of the animal model, dose and mode of administration of ethanol, time of assay after the last dose of ethanol, reaction time and sensitivity of the lipid peroxidation assay. Microsomes isolated from chronic ethanol-treated rats have been reported to display enhanced rates of lipid peroxidation [15–17], although a recent report found opposite results [18]. Here, the role of transition metals such as iron as a complicating variable in rates of lipid peroxidations between preparations from different treatments was evaluated. An additional problem was found as to what constitutes an appropriate control for the chronic ethanol-treated model.

2. MATERIALS AND METHODS

Male, Sprague-Dawley rats weighing about 135 g were pair-fed for 24 days a nutritionally adequate

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liquid diet in which ethanol provided 36% of total calories, protein 18%, fats 35% and carbohydrate 11% [19]. Pair-fed littermates consumed the same diet except that carbohydrate isocalorically replaced ethanol. Prior to the day of killing, the rats were allowed access to their respective diets ad libitum. Liver microsomes were prepared as described [4], washed once and suspended in 125 mM KCl. Microsomes were also prepared from rats fed Purina chow ad-lib.

Microsomal lipid peroxidation was assayed by determining the rate of production of malondialdehyde, or more specifically, the production of thiobarbituric acid-reactive components. Reactions were carried out under air at 37°C in test tubes utilizing a reaction system containing 50 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$, 0.4 mM $NADP^+$ and about 1 mg microsomal protein in a final volume of 1.0 ml. Tris buffer was used since rates of malondialdehyde production were about 3–4-fold greater than rates found with phosphate buffer. The reactions were initiated by the addition of a mixture of 10 mM glucose 6-phosphate plus 2 U glucose-6-phosphate dehydrogenase and terminated after 5, 10 or 15 min by the addition of 2 ml of a mixture containing 15% trichloroacetic acid, 0.25 N HCl, 0.375% thiobarbituric acid and 0.01% butylated hydroxytoluene. The antioxidant lowered blank values and prevented metal-catalyzed decomposition during the heating step [20]. Samples were boiled for 15 min in a boiling water bath, cooled and centrifuged for 10 min in a clinical centrifuge. The absorbance of the supernatant was determined at a wavelength of 535 nm. An extinction coefficient of 156 $mM^{-1} \cdot cm^{-1}$ was used to calculate the concentration of malondialdehyde [21]. All values were corrected for zero-time controls in which the acid mixture was added to the microsomes prior to the addition of the NADPH-generating system. The content of protein [22] and cytochrome P-450 [23] and the activity of NADPH-cytochrome *c* reductase [24] were determined as described. All values refer to mean ± SE.

3. RESULTS

The effect of chronic ethanol consumption on the rate of malondialdehyde production is shown in table 1. Malondialdehyde generation was linear over the 15 min reaction period and was increased by the addition of $FeCl_3$ to the reaction system. Compared to the pair-fed controls, the rate of malondialdehyde production by microsomes from chronic ethanol-treated rats was 2-fold greater in the absence of added iron, and about 4-fold greater in the presence of iron. These increases could be suggestive of an enhanced rate of lipid peroxidation by microsomes after chronic ethanol feeding. However, when rates of malondialdehyde production are compared to those of microsomes isolated from chow-fed controls, the rates with the alcohol-treated were actually lower, in both the absence and presence of added iron (table 1). Thus, rates of malondialdehyde production increased in the order chow-fed > chronic ethanol > pair-fed control.

In view of the importance of metals such as iron

Table 1

Effect of chronic ethanol treatment on microsomal production of malondialdehyde

Addition	Reaction time (min)	Rate of malondialdehyde production (nmol/mg microsomal protein)		
		Pair-fed	Alcohol	Chow
–	5	1.22 ± 0.4	2.58 ± 1.1	7.59 ± 1.7
	10	2.68 ± 1.2	6.37 ± 1.9	12.03 ± 1.8
	15	4.15 ± 2.3	9.38 ± 1.6	17.03 ± 2.7
25 μM $FeCl_3$	5	1.58 ± 0.5	6.38 ± 1.4	15.20 ± 0.2
	10	3.76 ± 1.4	15.04 ± 3.4	22.65 ± 0.9
	15	6.66 ± 2.3	21.64 ± 2.3	25.16 ± 2.4

Microsomal production of malondialdehyde was determined for the indicated reaction time periods in the absence or presence of added $FeCl_3$. Results are from either 5 (pair-fed and alcohol-treated) or 4 (chow) experiments

in promoting microsomal lipid peroxidation [6,7], it was anticipated that the different rates of malondialdehyde production observed for the three types of microsomal preparations could be a reflection of a varying endogenous microsomal content of iron. It should be mentioned that all buffers and the water used to prepare the solutions were passed through columns of Chelex 100 resin to remove extraneous metals. Desferrioxamine is a potent iron-chelating agent which has been shown to prevent iron-catalyzed microsomal production of hydroxyl radicals [25] as well as lipid peroxidation [26]. A titration curve of the effect of desferrioxamine on microsomal lipid peroxidation is shown in table 2. Whereas desferrioxamine inhibited the production of malondialdehyde in all microsomal preparations, the concentration of the chelator required to block lipid peroxidation in the three types of preparations was different. For example, in microsomes from the pair-fed controls, only 0.5 μM desferrioxamine was required to reach a low, steady-state level of malondialdehyde production. However, in microsomes from the chronic ethanol-treated, about 2 μM desferrioxamine was required, while in microsomes from the chow-fed controls, about 5 μM desferrioxamine was necessary to produce substantial inhibition of microsomal lipid peroxidation.

Table 2

Effect of desferrioxamine on microsomal production of malondialdehyde

Concentration of desferrioxamine (μM)	Rate of malondialdehyde production (nmol/min per mg microsomal protein)		
	Pair-fed	Alcohol	Chow
0	0.27 ± 0.12	0.64 ± 0.19	1.20 ± 0.18
0.5	0.13 ± 0.01	0.41 ± 0.01	1.33 ± 0.40
2	0.13 ± 0.01	0.17 ± 0.01	1.02 ± 0.30
5	0.10 ± 0.01	0.11 ± 0.01	0.43 ± 0.10
10	0.12 ± 0.01	0.11 ± 0.01	0.27 ± 0.01
25	0.12 ± 0.01	0.10 ± 0.01	0.24 ± 0.10

Microsomal production of malondialdehyde was determined in the presence of the indicated concentrations of added desferrioxamine. Results are from 3 experiments

Table 3

Concentration curve for the effect of iron on microsomal production of malondialdehyde

Concentration of $FeCl_3$ (μM)	Rate of malondialdehyde production (nmol/min per mg microsomal protein)		
	Pair-fed	Alcohol	Chow
0	0.27 ± 0.12	0.64 ± 0.19	1.20 ± 0.18
10	0.44 ± 0.02	1.18 ± 0.02	2.61 ± 0.07
25	0.48 ± 0.14	1.50 ± 0.30	2.72 ± 0.09
50	0.55 ± 0.02	1.50 ± 0.02	3.24 ± 0.07
100	0.57 ± 0.02	1.57 ± 0.02	3.46 ± 0.07

Microsomal production of malondialdehyde was determined in the presence of the indicated concentrations of added $FeCl_3$. Results are from 4 experiments

The varying response to desferrioxamine suggested that the content of iron was different in the three types of microsomal preparations. It was, therefore, considered that the addition of excess iron might cause the rates of malondialdehyde production to become equivalent among the different preparations. A concentration curve for the effect of $FeCl_3$ on microsomal lipid peroxidation is shown in table 3. A concentration of 10 μM $FeCl_3$ appeared sufficient to stimulate lipid peroxidation in all preparations; the K_m for ferric stimulation of NADPH-dependent lipid peroxidation of washed microsomes was reported to be about 2 μM [27]. Of interest is the observation that even at 100 μM $FeCl_3$, the effective order of microsomal lipid peroxidation remained chow-fed > chronic ethanol > pair-fed control (table 3). Thus, excess iron did not obliterate the differences in malondialdehyde production among the three types of preparations.

In other experiments, it was found that rates of malondialdehyde generation by all three types of microsomal preparations were not affected by the addition of either superoxide dismutase or catalase. Contents of cytochrome P-450 were 0.65 ± 0.1, 1.28 ± 0.1 and 0.68 ± 0.1 nmol per mg protein for microsomal preparations from pair-fed, chronic ethanol-fed and chow-fed, respectively, while activities of NADPH-cytochrome *c* reductase were 157 ± 8, 186 ± 13 and 174 ± 24 nmol/min per mg protein, respectively.

4. DISCUSSION

Microsomes isolated from chronic ethanol-fed rats can display either elevated rates or depressed rates of malondialdehyde production depending on what is considered as the appropriate control. Compared to the typical pair-fed control, rates of microsomal lipid peroxidation are elevated whereas if comparison is made to chow-fed controls, lipid peroxidation is lower after ethanol feeding. The problem of an appropriate control for the chronic ethanol liquid diet model has long been recognized [19,28]. In a variable complex process such as lipid peroxidation, the issue of an appropriate control is probably magnified. The different rates of malondialdehyde generation did not appear to correlate with altered content of cytochrome P-450 or activity of NADPH-cytochrome *c* reductase. In fact, the pair-fed and the chow-fed controls had identical contents or activities of these two major microsomal enzymes although rates of lipid peroxidation differed by more than 4-fold. Conversely, although chronic ethanol treatment produced the well-known [29] increase in P-450 content, rates of lipid peroxidation were intermediate between the pair-fed and chow-fed controls. Carbon monoxide (2:1 with respect to O_2) did not inhibit malondialdehyde production in any of the microsomal preparations (not shown). The detailed studies of Aust and coworkers [6,7,26] have emphasized the importance of the reductase, and not-P-450, in microsomal lipid peroxidation.

The critical role of iron in catalyzing lipid peroxidation would suggest that variations of iron content in the different microsomal preparations could explain the varying rates of malondialdehyde production. The titration curve with desferrioxamine did indicate that more iron was present in microsomes from the chow-fed controls than the chronic ethanol-fed, which in turn, had more iron than microsomes from the pair-fed controls. Since identical procedures and buffers were used to prepare the microsomes, we have no explanation for the varying iron content, however, this is probably a major factor for consideration in studies comparing oxygen radical generation and lipid peroxidation between different treatments.

The results in table 3, however, show that other variables besides the content of iron also need to be considered when comparing rates of malondialdehyde production between different treatments. Even in the presence of 100 μM $FeCl_3$, which far exceeds the content of iron in the microsomal preparations (estimated to range between 1 and 10 μM based on the desferrioxamine titration curve; 1 mole desferrioxamine binds 2 mole equivalents of ferric), rates of malondialdehyde production still vary considerably between the three types of microsomal preparations. Whether these differences reflect the isozyme pattern of P-450, or altered vitamin E content of the microsomal membranes or the fatty acid composition of microsomal phospholipids remains to be determined. Concerning the latter, only fatty acids with 3 or more methylene-interrupted double bonds can ultimately yield malondialdehyde and variations in malondialdehyde production could reflect the lipid composition rather than the actual susceptibility to lipid peroxidation [20].

Malondialdehyde production by the various microsomal preparations was insensitive to either superoxide dismutase or catalase suggesting that the oxidant responsibe for initiation of lipid peroxidation was not the hydroxyl radical. Disassociation of lipid peroxidation from hydroxyl radical generation has been observed by others [6,7,26]. Shaw et al. [18] actually found an inverse relationship between lipid peroxidation and hydroxyl radical generation by microsomes from ethanol-fed rats. Taken as a whole, the variabilities associated with the different controls, the role and content of transition metals such as iron, the fatty acid composition of the membranes, and the lack of identification of the actual initiating oxidant species complicate a clear interpretation of the effect of chronic ethanol consumption on microsomal lipid peroxidation. These variables probably play important roles in contributing towards the divergent reports of the effects of ethanol on lipid peroxidation.

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