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THE EFFECT OF CHRONIC ALCOHOL FEEDING ON LIPID PEROXIDATION IN MICROSOMES: LACK OF RELATIONSHIP TO HYDROXYL RADICAL GENERATION

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Chronic alcohol feeding causes microsomal induction including increased generation of hydroxyl radicals. Ethanol induced liver injury may be mediated by lipid peroxidation for which hydroxyl radicals have been proposed as major mediators. Ethanol promotes lipid peroxidation when given acutely but also may serve as a hydroxyl radical scavenger. Therefore, we studied the acute and chronic effects of alcohol on microsomal lipid peroxidation and hydroxyl radical generation. Chronic alcohol feeding in rats increased microsomal generation of hydroxyl radicals but lipid peroxidation of endogenous lipid was inversely related to hydroxyl radical generation. Ethanol (50mM) had a slight inhibitory effect on hydroxyl radical production in peroxidizing microsomes, no effect on endogenous lipid peroxidation and enhanced the lysis of RBCs added as targets of peroxidation. Enhanced microsomal generation of hydroxyl radicals following chronic alcohol feeding is not an important mediator of lipid peroxidation.

The mechanism of alcohol-induced liver injury is still debated; recent evidence has revived the concept that lipid peroxidation is an important contributing mechanism (1-5). Chronic alcohol feeding induces hepatic microsomes (6,7) and increases their generation of hydroxyl radicals (8). Some evidence suggests that hydroxyl radicals may be the most important free radicals initiating lipid peroxidation in microsomes (9-11) and thus, enhanced hydroxyl radical production could be a mechanisms by which chronic alcohol feeding promotes peroxidation. Paradoxically, acute ethanol administration promotes microsomal lipid peroxidation (2) but also acts as an hydroxyl radical scavenger (12,13). The latter effect would inhibit lipid peroxidation. Other evidence, however, suggests that superoxide or singlet oxygen rather than hydroxyl radicals may initiate lipid peroxidation (14-16). Furthermore, scavenging of hydroxyl radicals

Abbreviations used are: DMSO, dimethylsufoxide; NADP, β nicotinamide adenine dinucleotide; HCHO, formaldehyde; TCA, trichloroacetic acid; MDA, malondialdehyde; RBC, red blood corpuscle; SOD, superoxide dismutase.

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by ethanol produces acetaldehyde (12,13) which itself could promote peroxidation by depleting protective compounds.

The relationship between increased hydroxyl radical production by microsomes after chronic alcohol feeding and lipid peroxidation were studied in the rat.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats were pair-fed a nutritionally adequate liquid diet containing protein as 18% and ethanol as 36% of total calories (alcohol diet) or the identical diet with ethanol substituted isocalorically by carbohydrate (control diet) (17). Animals were sacrificed by decapitation after 4 weeks of pair feeding following an overnight fast. For the studies measuring red blood cell lysis, microsomes were obtained from rats fed Purina Chow and libitum.

Preparation of Microsomes: Following sacrifice livers were perfused via the portal vein with iced 1.15% KCl, homogenized in 4 volumes of KCl and spun at 10,000 G for 30 minutes. Microsomes were harvested by spinning at 105,000 G for 60 minutes. The pellet was washed with 1.15% KCl, resuspended and spun again.

Microsome Incubations: Incubations were in 5ml volumes in capped 25ml Erlenmeyer flasks at 37°C. Incubations consisted of .1M sodium phosphate buffer pH 7.4, and a/NADPH generating system (isocitrate dehydrogenase 0.4 units/ml, isocitrate (8mM), NADP (0.4mM) and $MgCl_2$ (6mM)). Microsomal protein was measured by the method of Lowry et al (18). Reaction mixtures contained 1mg/ml microsomal protein.

Lipid peroxidation from endogenous lipid was assessed by measuring MDA in 1 ml aliquots every 5 minutes for 15 minutes. Production of hydroxyl radicals was measured by adding DMSO (30mM) to reaction mixtures and measuring HCHO formation. Incubations were performed with microsomes from pair-fed animals in the presence of ethanol (50mM), azide (5mM) EDTA (1mM) and SOD (50µg/ml).

Additional incubations were carried out with microsomes from chow-fed animals for a 1 hour period to which RBCs were added. The rate of lysis was measured by the appearance of hemoglobin in the media.

Measurement of MDA: Net production of MDA was measured over 15 minutes. Proteins were precipitated by the addition of 0.1ml of 2% deoxycholic acid and 0.2 ml of 20% w/v TCA. The samples were centrifuged at 14,000 G for 10 minutes and the supernatant mixed with an equal volume of 0.75% 2-thiobarbituric acid and incubated at 100°C for 10 minutes. Samples were cooled to 25°C and the absorbance read at 409nm. Bis malondialdehyde tetraethylacetal served as a standard. MDA could not be measured in the presence of DMSO because of color interference.

Measurement of HCHO from DMSO: Microsomal hydroxyl radical generation was assessed by adding DMSO (30mM) to the incubation and measuring HCHO at 5 minute intervals for 15 minutes as described by Klein et al. (1981) (19). Protein was precipitated by addition of 0.3 ml of 30% trichloroacetic acid. HCHO was measured by the Nash reaction (20).

RBC Hemolysis: To further assess the relationship between hydroxyl radical production and lipid peroxidation, incubations were conducted with RBCs added as targets. Heparinized blood was obtained fresh from the same normal volunteer. RBCs were washed twice (suspended in 10 volumes of 0.9% saline and spun at 10,000 G for 5 minutes at 5°C) and added to microsomal incubations to a final hematocrit of 1%. Aliquots were taken at 15 minute intervals. Lysis was stopped by addition of $MnCl_2$ (final concentration 1mM) according to the method of Pfeiffer and McCay (21). Samples were spun at 10,000 G for 5 minutes and the absorbance of the supernatants at 545nm was measured in a spectrophotometer. Percent hemolysis was calculated by comparison to the absorbance of RBCs completely hemolyzed in distilled water.

Chemicals and Reagents: Isocitric dehydrogenase (pig heart Type I), D,L-isocitric acid (trisodium salt, Type I), β-nicotinamide adenine dinucleotide (NADP) (yeast, monosodium salt), superoxide dismutase (bovine blood, type I) and dimethyl sulfoxide (grade I) were obtained from Sigma Chemical Company, St. Louis, Mo. 2-thiobarbituric acid was obtained from the Eastman Kodak Company, Rochester,

N.Y.. Bis-malondialdehyde tetraethylacetal was obtained from Merck-Schuchardt, Munich, Germany. Other chemicals were of reagent grade of better.

Statistics: Data were expressed as the mean ± standard error of the mean. Differences between groups were determined by the Student's paired t-test.

RESULTS

The effect of chronic alcohol feeding on hydroxyl radical production in microsomes is shown in Table 1. Chronic alcohol feeding increased the generation of HCHO from DMSO approximately 2-fold. EDTA (1mM) markedly enhanced HCHO production and this increase was greatest in the presence of azide. Addition of SOD (50µg/ml) decreased HCHO production by approximately 50% in both alcohol-fed animals and controls in the absence but not in the presence of azide (Table 1). The addition of ethanol decreased HCHO production in alcohol-fed animals and controls (30-50%) but these differences failed to attain significance.

Malondialdehyde (MDA) production from endogenous lipid in microsomal incubations from alcohol-fed animals was significantly lower than that in pair-fed controls (Table 1). Ethanol had no effect on MDA production. EDTA virtually abolished MDA production and SOD increased production (Table 1). There was a statistically significant negative correlation between production of HCHO from DMSO and endogenous production of MDA (n=16, r=-0.734, $p<0.001$).

TABLE 1. Effect of chronic alcohol feeding on formaldehyde (HCHO) production from dimethylsulfoxide (DMSO) and endogenous production of malondialdehyde (MDA) (in the absence of DMSO).

INCUBATION	Formaldehyde production from added DMSO (30mM)		Malondialdehyde production from endogenous lipids	
	HCHO (nmoles/hr/mg protein)		MDA (nmoles/hr/mg protein)	
	Alcohol-Fed (n=6)	Control (n=6)	Alcohol-fed (n=6)	Control (n=6)
NADPH Gen System	103.2±27.9^{x}	52.2±12.3	32.5±6.9^{xxx}	101.8±11.9
" + Azide (5mM)	100.9±27.4^{x}	46.7±12.0	44.1±6.9	73.4±15.5
" + Ethanol (50mM)	71.4±14.5^{x}	25.5±9.9	40.0±10.6	100.6±17.4
" + Ethanol + Azide	69.1±12.5^{x}	48.6±10.1	45.9±12.3	74.2±16.7
" + SOD (50µg/ml)	50.6±7.8^{xx}	26.5±8.8	140.1±47.3	155.8±36.0
" + SOD + Azide	78.6±19.5	53.4±13.1	146.0±38.2	129.6±33.1
" + EDTA (1mM)	197.2±19.5	149.4±33.1	13.2±3.3	13.8±5.4
" + EDTA + Azide	274.1±57.0	239.2±59.1	11.0±3.6	15.2±5.0

(vs pair-fed control)
x p <.05
xx p <.02
xxx p <.01

TABLE 2. Lysis of red blood cells in peroxidizing microsomes. The results paralled those for MDA generation with the exception that the addition of ethanol significantly increased RBC lysis.

INCUBATION	RBC LYSIS (%)		
TIME	15 minutes	30 minutes	60 minutes
NADPH Gen. System	6.8±1.8	42.7±18.1	87.5±3.6
" + Azide (5mM)	6.6±1.6	7.1±2.1	67.1±5.4
" + Ethanol (50mM)	15.4±6.6	85.5 ± 3.7^{xx}	92.1±2.8
" + Ethanol + Azide	5.9±1.6	34.2 ± 8.0^{xxx}	69.8±4.2
" + SOD (50 µg/ml)	36.3 ± 10.9^{x}	91.2 ± 4.0^{xxx}	91.1±8.4
" + SOD + Azide	34.3 ± 11.5^{x}	77.3 ± 1.7^{xxxx}	75.6±5.0
" + EDTA (1mM)	0.4±0.3	1.3 ± 0.8^{xx}	5.0±1.6
" + EDTA + Azide	0.6±0.5	0.5 ± 0.3^{xxx}	4.9±1.8

p vs NADPH generating system (±Azide) without other additions
x p <0.05
xx p <0.02
xxx p <0.01
xxxx p <0.001

The lysis of RBCs in microsomal incubations (Table 2) paralleled the findings of MDA production. EDTA which virtually abolished MDA production prevented RBC lysis. The addition of SOD increased the rate of lysis at 15 minutes. Ethanol tended to increase RBC lysis at 15 minutes and significantly increased lysis at 30 minutes. Incubation of RBCs in phosphate buffer alone resulted in <2% hemolysis at 1 hour and was not significantly affected by the addition of either ethanol (50mM) or acetaldehyde (50µM).

DISCUSSION

This study reveals that chronic alcohol feeding increases the capacity of hepatic microsomes to generate hydroxyl radicals but that there is a striking dissociation between hydroxyl radical production and microsomal lipid peroxidation. These results also demonstrate that ethanol is a weak hydroxyl radical scavenger, has little effect on microsomal lipid peroxidation in vitro and increases the lysis of RBCs added as target cells.

The finding that ethanol-induced microsomes have an increased capacity to generate hydroxyl radicals confirms previous observations using DMSO as well as other scavengers (8). The relationship between hydroxyl radicals and microsomal lipid peroxidation is controversial (9-11, 14-16). Our results support the position that free radicals other than hydroxyl radicals are most important in

microsomal lipid peroxidation (14-16). Hydroxyl radicals may be interconverted to other free radicals (superoxide and singlet oxygen) by interactions such as the Haber-Weiss and Fenton reactions. Our incubations using phosphate buffer may have maximized of the production of hydroxyl radicals because of the presence contaminating iron. Indeed, tight chelation of iron with desferrioxamine markedly inhibits hydroxyl radical formation (22). It is possible that hydroxyl radicals generated by ethanol-induced microsomes could under other conditions be interconverted to an excess of other species promoting peroxidation.

Increased MDA production from microsomes following chronic alcohol feeding has been previously demonstrated but the studies were not comparable to ours. In one study an acute dose of ethanol was given to animals chronically fed alcohol prior to sacrifice (23); thus, acute and chronic effects were mixed. This fact is especially significant since it has been demonstrated that acute ethanol administration to rats chronically fed ethanol will promote lipid peroxidation (2). In the other study precise dietary control was not maintained (24).

Previous studies have demonstrated that the lysis of RBCs in microsomal incubations is due to short lived free radicals (21). In our studies the rate of lysis of RBCs was altered by factors that affect lipid peroxidation. Indeed, the rate of lysis paralleled the generation of MDA in these incubations. An unexpected finding was the enhanced lysis of RBCs when ethanol was added to the incubations and the lack of effect of ethanol or acetaldehyde in the absence of microsomes. Thus, ethanol or its metabolite acetaldehyde do not directly augment lysis. They may, however, play a secondary role by altering the physical state of the RBC membrane or by interfering with protective mechanisms.

In conclusion, chronic alcohol feeding increases microsomal generation of hydroxyl radicals in vitro but lipid peroxidation does not appear to be related to hydroxyl radical production per se. Ethanol may act as an hydroxyl radical scavenger (albeit a weak one), has little effect on microsomal peroxidation of endogenous lipid and enhances the lysis of RBCs by microsomes.

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