Glutathione Recycling is Attenuated by Acute Ethanol Feeding in Rat Liver

The mechanism for ethanol-induced oxidative stress has been disputed because of the controversies on modulation of radical generating and scavenging activities by ethanol. In the present work, we attempted to clarify the acute effect of ethanol on the radical generating system as well as the radical scavenging system. For that purpose, chow-fed rats were given ethanol (5 g/kg) or isocaloric glucose solution by intragastric intubation and placed at 32℃ for 6 hr. Acute ethanol administration enhanced the expression of cytochrome P450 II E1 (CYP II E1) in the liver and attenuated the activities of hepatic glutathione peroxidase (GPx) and reductase (GR). It also caused a significant increase in the level of hepatic thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation. On the other hand, acute ethanol feeding had no effect on the activities of catalase, xanthine oxidase (XO), glutathione transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH). From this result, it is suggested that acute ethanol administration causes the oxidative tissue damage by CYP II E1-associated radical generation and the decreased radical scavenging function due to the reduced activities of hepatic glutathione recycling system such as GPx and GR. ***(JKMS 1997; 12: 316~21)***

Key Words : *Cytochrome P450 II E1, Thiobarbituric acid reactive substances (TBARS), Glutathione peroxidase, Glutathione reductase, Ethanol*

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INTRODUCTION

Ethanol toxicity is derived either directly from ethanol *per se* and its metabolite, acetaldehyde, or indirectly from the metabolic sequelae of ethanol oxidation such as the decreased ratio of cytoplasmic NAD^+/NADH and the involvement of reactive oxygen species (1). For the ethanol-induced generation of free-radical species, two systems have been postulated to be responsible. One includes cytochrome P450's and NADPH reductase-dependent microsomal oxidizing system (2) and the other one involves xanthine oxidase (3). Of the microsomal P450's, the ethanol-inducible isoform, P450 II E1, is believed to induce oxidative damage (4).

Regarding the role of lipid peroxidation in the pathogenesis of ethanol-related tissue damage, many controversies exist (5~10). An acute ethanol load was reported by Comporti and colleagues (5) to increase liver lipid peroxidation 1 to 12 hours after oral intubation. However, Hashimoto and Recknagel (7) reported no increases in conjugated diene in rat liver mitochondrial or microsomal lipid following a single dose of ethanol administration. The reported discrepancies might have been due to variations in experimental design (mode of administration, dose, animal species, gender and age). And the oxidative tissue damage in the liver of ethanol-ingesting animal is known to vary according to the balance between the potential to form activated oxygen radicals and the capacity to remove them and lipid peroxides.

Many experimental studies were conducted to delineate the effect of ethanol administration on the major hepatic antioxidative enzymes and substrates, but the reported changes in hepatic antioxidative enzyme activities after ethanol administration appear highly contradictory (11~15). On the one hand, most reports focused on the changes in the activities of catalase and GPx as one of the scavenging mechanisms for ethanol-induced radicals (16~18). On the other hand, the depletion of glutathione, regardless of the reduced or the oxidized form, by ethanol was illustrated (19~25). However, there are few reports on the importance of glutathione recycling, of which the efficiency might play an important role in ethanol-induced oxidative stress.

Therefore, in order to elucidate the role of ethanol-induced lipid peroxidation and its relationship to gen-

eration and scavenging of oxygen radicals in the liver, chow-fed Sprague Dawley rats were given ethanol (5 g/kg) by garbage and placed at 32℃ for 6 hrs. Then we examined the various parameters of oxidative stress and antioxidative defense system.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 140~180 g were used. All animals had free access to standard rat chow (Samyang Co., Seoul, Korea) and water. The rats were kept at 22~25℃ with 12 hours light/dark cycle.

Experimental design

After an overnight fast, rats were given ethanol (5 g/kg, 30% wt/vol) or isocaloric glucose (8.75 g/kg, 52.5% wt/vol) in water intragastrically and placed in a room maintained at 32℃. A warm environment prevents the hypothermia associated with a large dose of ethanol administration and permits better reproducibility of the lipid peroxidation produced by an acute ethanol dose (8). Six hours after ethanol feeding, the animals were sacrificed by decapitation and livers were excised, rinsed, blot dried and weighed.

Sample preparation

Livers were homogenized with glass/Teflon homogenizer (S63C, Tri-R Instruments, USA) and the homogenate was used to prepare cytosol and microsomal fractions. Liver homogenate was prepared with 9 vol. of ice cold 1.15 % KCl with 0.2 mM PMSF and 1 mM DTT using 5 up and down strokes and filtered through 3 layers of gauze to remove connective tissues. Then it was spun at 800 g for 10 min (H50E-TR, Hanil Centrifuge Co., Korea) to get rid of cell debris and nuclei, and the supernatant, a suspension of mixed and preserved organelles, was centrifuged at 13,200 g for 10 min (VS-15000CF, Vision Scientific Co., Korea). The postmitochondrial (13,200 g) supernatant was centrifuged again at 105,000 g for 60 min (TL-100, Beckman Co., Korea) to prepare microsomes and cytosol fractions. The pellet (microsomes) resuspended in the same buffer and the supernatant fraction (cytosol) were recentrifuged at 105,000 g for an additional 30 min to reduce cross contamination. Washed microsomes resuspended in the same buffer and the cleaner supernatant taken as cytosol fraction were stored at -70℃ in small aliquots. The whole procedure was carried out at 0~4℃.

Western blotting

SDS polyacryamide gel electrophoresis was run using 10% acrylamide gel with 30 μg of microsomal protein in each well and bands were transferred to nitrocellulose paper. Enhanced chemiluminescence method was adopted to monitor the intensity of CYP II E1 bands using mouse monoclonal antibodies as primary antibodies (were kindly donated by Dr. Byung June Song from NIAAA) and peroxidase-conjugated anti mouse immunoglobulins (Vector Labortories, Inc., Burlingame, CA) as secondary antibodies.

Biochemical analysis

Thiobarbituric acid reactive substances

The content of 2-thiobarbituric acid reactive substances (TBARS) in the liver homogenate was measured as a marker of lipid peroxidation by the method of Uchiyama and Mihara (26) using 1, 1, 3, 3-tetraethoxypropane (TEP) as a standard. Absorbance of butanol phase was read at 535 and 520 nm against n-butanol (Uvikon 930, Kontron Instruments, Switzerland). The difference between two readings was used in calculation of TBARS content against that of 1, 1, 3, 3-TEP.

Carbonyl groups in protein

The extent of protein carbonylation was determined using 2,4-dinitropheylhydrazine reaction according to the method of Levine et al. (27). Insoluble materials were removed by centrifuging briefly in the Eppendorf tubes and the absorbance of supernatant was read. A molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ at 370 nm was used to calculate the carbonyl group content.

Enzymatic assays

XO and xanthine dehydrogenase (XDH) activities were measured using a slight modification of the methods descibed previously (28). The enzyme reaction mixture contained 0.2 mM xanthine, 0.1 M Tris-HCl buffer (pH 8.1), 0.1 mM EDTA and 150 μl of cytosol fraction in a final volume of 1 ml. Activity was monitored by reading optical densities at 300 and 340 nm, in the absence or presence of 0.5 mM NAD^+ at 30℃.

GPx was assayed in 50 mM Tris-0.1 mM EDTA buffer (pH 7.6) at 37℃ by measuring the oxidation of NADPH at 340 nm with cumene hydroperoxide as a substrate (29). GR was assayed in 0.2 M potassium phosphate buffer, pH 7.0 by monitoring the oxidation of NADPH at 340 nm with GSSG (the oxidized form of glutathione) as a substrate (30). Assay of GST activity was performed in phosphate buffer, pH 6.5, using 1-

chloro-2,4-dinitrobenzene as a substrate and the absorbance change was recorded at 340 nm (31). The activity was calculated using extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. Liver catalase activity was assayed in the homogenate at 20℃ according to the method of Aebi (32). H_2O_2 disappearance was monitored kinetically at 240 nm and the activity was expressed in rate constant (*k*/mg protein) as recommended by Aebi (32). G6PDH activity was measured at 25℃ according to Löhr and Waller (33).

Proteins

Proteins were assayed with the Bradford method (34) using bovine serum albumin as a standard.

Statistical analysis

Data were expressed as mean±standard error of the mean (SEM). The statistical significance of the difference between the group means was determined by the Student's *t*-test.

RESULTS

Effect of ethanol on the generation of reactive oxygen species

Acute ethanol consumption resulted in an increase of CYP II E1 (Fig. 1) implying its possible involvement in activated oxygen radical formation during ethanol metabolism. However, there was no significant difference in the activity of XO between these two groups (Table 1).

Effect of ethanol on the oxidative status

The effect of acute ethanol consumption on the level of TBARS and protein carbonyl group is shown in Table 2. While the amount of TBARS in the liver was significantly increased ($p < 0.05$) by acute ethanol treatment, the level of carbonyl group content was not different between ethanol-fed and control animals.

Table 1. Effect of acute ethanol administration on xanthine dehydrogenase (XDH) and xanthine oxidase (XO) activities and XDH/XO ratio

Group	XDH	XO	XDH+XO	XDH/XO ratio
	(nmoles/min/mg protein)			
Control	20.5±1.1[a]	0.40±0.04	20.9±1.1	52.5±4.1
Ethanol	21.9±1.2	0.40±0.03	22.3±1.2	57.2±4.6
p[b]	NS	NS	NS	NS

a) Values are expressed as mean±SEM of 6 rats.
b) Statistical significance was determined by Student's *t*-test.

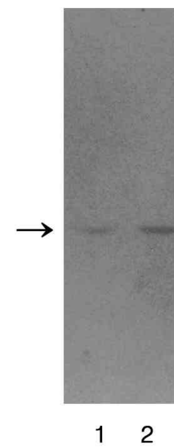

Fig. 1. Effect of acute ethanol administration on cytochrome P450 II E1 induction in rat liver microsomes. Lanes 1, control group ; and lanes 2, ethanol group.

Table 2. Effect of acute ethanol administration on ethanol-induced liver damage

Indicators of ethanol-induced liver damage	Group: Control	Group: Ethanol	p[a]
TBARS (nmoles/mg protein)	2.39±0.66	4.78±0.76	<0.05
Carbonyl group content (nmoles/mg protein)	2.46±0.10	2.34±0.13	NS[b]

a) Values are expressed as mean±SEM of 6 rats. Statistical significance was determined by Student's *t*-test.
b) not significant.

Effect of ethanol on the antioxidative enzymatic system

Table 3 presents the activities of catalase, GPx, GR, GST and G6PDH in the liver of control and ethanol-treated animals. The activities of hepatic GPx and GR in ethanol-treated rats were decreased significantly com-

Table 3. Effect of acute ethanol administration on free radical scavenging enzyme activities in liver

Radical scavenging enzymes	Group: Control	Group: Ethanol	p[a]
Catalase (*k*/mg protein)	1.15±0.10[c]	1.17±0.14	NS
GPx[b] (nmoles/min/mg protein)	438±23	389±18	<0.01
GR (nmoles/min/mg protein)	152±9	127±5	<0.05
G6PDH (nmoles/min/mg protein)	9.66±0.55	9.28±0.50	NS
GST (nmoles/min/mg protein)	1,742±94	1,705±75	NS

a) Statistical significance was determined by Student's *t*-test.
b) Abbreviations : GPx, glutathione peroxidase; GR, glutathione reductase ; G6P-DH, glucose-6-phosphate dehydrogenase ; GST, glutathione transferase.
c) Each value represents the mean±SEM of 6 rats.

pared to those of controls($p<0.01$, $p<0.05$, respectively). On the other hand, acute ethanol administration did not cause any significant changes in the hepatic activities of catalase, G6PDH and GST.

DISCUSSION

Acute ethanol consumption resulted in an increased expression of CYP II E1 (Fig. 1). This pathway of metabolism is likely to play an important role in the generation of reactive oxygen species during ethanol oxidation by hepatic microsomes, since it reduces dioxygen to oxygen-derived radicals and H_2O_2 (2, 35). Actually a correlation between the production of these oxygen derivatives (O_2^-, H_2O_2) and the amount of CYP II E1 was observed in the hepatic microsomal samples of variously treated rats (4).

Another mechanism for free radical production after ethanol intake would be an association with XO. Normally, the activity of NAD^+-dependent XDH, which does not produce superoxide, prevails over that of XO in the liver. However, NADH, which is increased as a consequence of ethanol metabolism *in vivo* can inhibit the dehydrogenase activity. And the consequential shift in the metabolism of hypoxanthine and xanthine to XO pathway from XDH pathway would then favor production of oxygen radicals and hydrogen peroxide. However, in the present study, there was no significant change in XO and XDH activities under our experimental conditions (Table 1). Thus, the ethanol-inducible cytochrome P450s seemed to play more important role than XO pathway in the generation of ethanol-induced reactive oxygen species which could damage proteins, carbohydrates, nucleic acids and polyunsaturated fatty acids (2, 35~39). Their reactions with polyunsaturated fatty acids would have induced lipid peroxidation and the produced lipid hydroperoxides decomposed to short-chain hydrocarbons and very reactive aldehydes such as malondialdehyde, 4-hydroxynonenal, etc. Accordingly, acute ethanol treated group had significantly higher TBARS level in liver as compared with that in control group (Table 2). As mentioned earlier, hepatic CYP II E1 might have been responsible for generation of various free radical species and the resulting oxidative tissue damage.

The oxidative stress, however, affects cellular integrity only when the antioxidative mechanisms are no longer able to cope with the free radical generation. Therefore, we analyzed the capacity of antioxidative defense system by monitoring the activities of various enzymes involved in the elimination of activated oxygen radicals in rat livers. The decrease in the activity of liver GPx after acute ethanol treatment would contribute to the increase in hepatic peroxide level(Table 3). In the study of Morton and Mitchell(40), there was a GPx activity decrease similar to our result in ethanol-fed rat, suggesting a reduced capacity to scavenge radicals. On the other hand, it is hard to find reports on the activity of GR, a cytosolic NADPH-dependent enzyme which regenerate GSH (the reduced form of glutathione) from GSSG following ethanol administration. Nonetheless, we observed a significantly decreased GR activity by ethanol feeding (Table 3). The decreases in the activities of both GPx and GR by acute ethanol administration suggest a limitation in glutathione availability in the liver of ethanol-fed rats. Since the redox cycle of glutathione is important for the efficiency of glutathione-utilizing detoxification, the inhibition on the glutathione recycling enzyme activity would exacerbate the ethanol-induced oxidative stress.

In addition, it has been already known that acute ethanol administration causes a significant depletion of GSH content in the liver, with a concomitant decrease of GSSG content (19~25). The mechanisms proposed for this glutathione lowering by ethanol were the increased glutathione oxidation resulting from the enhanced generation of oxidizing radicals (21) and the reduced hepatic glutathione synthesis as well as the increased efflux of glutathione from the liver (22, 23). With the raised demand for hepatic GSH during oxidative stress, the depletion of GSH following acute ethanol intoxication might aggravate the outcome of lipid peroxidation. And therefore, the reduction in total glutathione contents as well as in glutathione recycling enzymic activities such as GPx and GR by acute ethanol treatment would account for the ethanol-induced oxidative tissue damages.

In contrast, there was no change in the activity of G6PDH following acute ethanol administration(Table 3). Considering the physiological role of the enzyme in supplying NADPH for cytosolic GR and microsomal mixed-function oxidase system, the steady G6PDH activity in acute ethanol intoxication would not influence the activities of radical generation and scavenging enzymes. Moreover, GST, the major detoxifying enzyme in the liver, as well as catalase, was not affected by acute ethanol intoxication for its activity.

In summary, acute ethanol administration enhanced the hepatic level of CYP II E1, the microsomal free-radical generation system, which contributed to ethanol-induced oxidative tissue damage in one way. And the attenuated activities of glutathione redox cycle enzymes such as GR and GPx by acute ethanol feeding besides the depletion of total glutathione content, appeared to be responsible for ethanol-induced oxidative stress in another way.

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