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Effects of amino acids on ethanol metabolism and oxidative stress in the ethanol-perfused rat liver

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ABSTRACT: **One mechanism of free-radical production by ethanol is suggested to be through the intracellular conversion of XDH to XO by increased ratio of NADH to NAD. The major mechanism for physiological compensation of cytosolic NADH/NAD balance is the malate/aspartate shuttle. Therefore, it is important to develop the method to improve the efficiency of malate/aspartate shuttle in ethanol metabolism. In the present study, various amino acids and organic acid involved in the shuttle were tested for their functional efficiency in modulating shuttle in the ethanol-perfused rat liver. The rate of ethanol oxidation in the liver perfused with aspartate alone or aspartate in combination with pyruvate, respectively, was increased by about 10% compared to control liver, but not in the tissues perfused with glutamate, cysteine or pyruvate alone. Though glutamate, cysteine and pyruvate did not affect the ethanol oxidation significantly, they showed some suppresive effect on the ethanol-induced radical generation monitored by protein carbonylation analysis. Among the tested components, aspartate is confirmed to be the most efficient as a metabolic regulator for both ethanol oxidation and ethanol-induced oxidative stress in our perfusion system. These effects of aspartate would result from NAD recycling by its supplementation through the coupled aspartate aminotransferase/malate dehydrogenase reactions and the malate-aspartate shuttle.**

Keywords: ethanol oxidation, amino acids, oxidative stress, xanthine oxidase, liver perfusion

INTRODUCTION

It is generally considered that ethanol oxidation in liver is limited by the rate of reoxidation of cytosolic NADH (Meijor *et al.*, 1975). The implication of translocation of reducing equivalents and the specific role of the malate-aspartate shuttle has been emphasized for cellular balance of NADH/NAD (Nordmann *et al.*, 1975: Cederbaum *et al.*, 1977: Chen *et al.*, 1989). This shuttle is considered as the main pathway for transferring reducing equivalents originated from the cytosol during ethanol oxidation into mitochondria. Recently, our study showed that, in the ethanol-perfused rat liver, there is a dose-dependent increase in the rate of ethanol oxidation with infusion of asparate, one of intermediates of the shuttle (Park, 1993: Park *et al.*, 1995). This suggests that the regeneration of NAD by the augmentation of malate-aspartate shuttle and coupled reaction of malate dehydrogenase (MDH) and alcohol dehydrogenase (ADH) may facilitate ethanol oxidation. Moreover, it was suggested that the increased ratio of NADH to NAD during ethanol oxidation would be involved in ethanol-induced oxidative stress. In particular, the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) by NADH has been reported to facilitate the generation of free radicals (Kato *et al.*, 1990; Younes *et al.*, 1987). Therefore, lowering the level of cytosolic NADH would be important not only to increase ethanol oxidation but also to attenuate the ethanol-induced oxidative stress.

Since the intracellular substrates of the shuttle are consumed competitively by other metabolic pathway for utilization, supplementation of shuttle intermediates would be required for optimal operation of the shuttle under extraordinary physiological conditions, such as when excess reduced equivalents are generated by overdose of ethanol intake. In the present study, vari-

ous amino acids and organic acid as intermediates or substrates involved in the malate-aspartate shuttle were tested for their functions in modulating the shuttle efficiency as well as for their effects on ethanol oxidation and ethanol-induced oxidative stress.

MATERIALS AND METHODS

Liver Perfusion

Male Sprague-Dawley rats weighing 240±10 g were used for all experiments. A perfusion technique *in situ* was a modification of those described previously (Reinke *et al.*, 1982; Deaciuc *et al.*, 1992). The portal vein and inferior vena cava were cannulated. Basic perfusate was consisted of a standard Krebs-Ringer bicarbonate buffer containing 0.1% glucose at pH 7.4. For 1 hr prior to and throughout the perfusion, the perfusate was gassed with a mixture of 95% O_2 and 5% CO_2, stirred at a hydrostatic pressure of 20 cm of water to ensure complete saturation with oxygen and its flow rate was 2 ml/min. Perfusion for each amino acid was carried out for 40 min.

Sample Preparation

After perfusion, livers were excised and kept frozen at -70°C. In order to analyze for lipid peroxidation and protein carbonylation, subcellular fractions were prepared from 10% (w/v) liver homogenates in 0.15 M NaCl by differential centrifugation. Meanwhile, 25% (w/v) liver homogenate was prepared in 0.1 M Tris-HCl buffer (pH 8.1) containing 0.1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine hydrochloride, 1 mM aprotinin and 1 mM dithiothreitol and used for XDH and XO assay.

Analytical procedure and enzyme assay

A series of experiments were performed to determine the rate of ethanol oxidation in response to various amino acids. Ethanol and amino acids were separately infused into the liver. Effluent samples were collected every 4 minutes for 40 minutes with fraction collector. The amount of ethanol was measured by gas chromatography (Hewlett-Packard, U.S.A). As the internal standard, t-butanol (2-methyl-2-propanol 0.05 mg/ml) was added to each sample. The temperature for injection and detection was 200°C while the column oven was maintained at 70°C. The resulting elution profile was analyzed with an integrator (HP 3396A, Hewlett-Packard, U.S.A.). Lipid peroxidation was assessed by the measurement of thiobarbituric acid-reactive substance (TBARS) (Esterbauer *et al.*, 1990). The fractions were added to an equal volume of thiobarbituric acid (TBA) reagent (0.375% TBA, 15% trichloroacetic acid, 0.25 M HCl). After mixing, samples were heated for 15 min in boiling water bath and centrifuged at 800 g for 10 min after cooling. The supernatant was monitored for its absorbance at 535 nm, and its TBARS (malondialdehyde, MDA) content was determined by using an extinction coefficient of 156,000 M^{-1}. cm^{-1}. Extent of protein carbonylation was determined by 2, 4-dinitrophenylhydrazine (DNPH) reaction (Levine *et al.*, 1990). The fractions were treated with 10% trichloroacetic acid solution. After 5 min incubation at 4°C, the samples were centrifuged at 800 g for 5 min. The 0.2% DNPH solution in 2 M HCl was added to the precipitates and mixed thoroughly. After 1 h incubation in a shaking incubator at room temperature, the protein was heated in 10% trichloroacetic acid and collected again by centrifugation. A tissue blank for each sample was prepared by treating with 2 M HCl instead of DNPH. The precipitate was dissolved in an equal volume of 6 M guanidine-HCl in 20 mM sodium phosphate buffer (pH 6) after washing twice with an ethanol-ethylacetate mixture (1:1, v/v). Insoluble materials were removed by centrifugation in the microcentrifuge for 2.5 min. A molar absorption coefficient of 22,000 M^{-1}. cm^{-1}. at 370 nm was used to calculate the carbonyl content. XDH and XO activities were measured using a slight modification of the methods described previously (Waud *et al.*, 1976; Kato *et al.*, 1990). The enzyme reaction mixture contained 0.2 mM xanthine, 0.1 M Tris-HCl buffer (pH 8.1), 0.1 mM EDTA and 100 µl of sample fraction in a final volume of 1 ml. Activity was monitored by reading optical densities at 295 and 340 nm, in the absence or presence of 0.5 nM NAD at 30°C. One unit (U) of both enzyme activity is defined as the amount of enzyme required to produce 1 µmol of NADH or 1 µmol of uric acid per min, respectively. Specific activity was calculated as milliunit (mU) per mg protein. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Effects on ethanol metabolism

The diminished amount of ethanol in the effluents in

response to infusion of various amino acids was expressed as a percentage compared to that of the control groups (Table 1). Each value is the average of three to four individual rats, perfused at a final concentration of 2 mM amino acid with 0.1% ethanol. The rate of ethanol oxidation in livers perfused with aspartate alone or aspartate in combination with pyruvate, respectively was increased by about 10% compared to that of single ethanol-perfused livers ($P<0.05$). However, this increase was not found in livers perfused with glutamate, cysteine or pyruvate alone, showing the similar rate to that of the control in ethanol oxidation.

Lipid peroxidation and protein carbonylation

The effects of amino acid treatment on the ethanol-induced generation of TBARS in the ethanol-perfused rat liver tissues were summarized in Table 2. The liver perfused only with buffer for 40 min was served as the control. The level of TBARS was about 2.7 fold higher in the liver perfused with 0.1% ethanol than that in control. This increase was slightly reduced by simultaneous perfusion with either glutamate or cysteine. However, the level of TBARS was reduced approximately to 60% of the control after aspartate and/or pyruvate treatment ($P<0.05$). A similar pattern was observed in ethanol-induced protein carbonylation (Table 2). However, the inhibitory effect of glutamate and cysteine on the ethanol-induced protein carbonylation was more marked than that in the ethanol-induced generation of TBARS.

Table 1. Effect of various amino acids on ethanol metabolism in the perfused-rat liver

	Ethanol in vascular effluents (% of control)
EtOH	100
EtOH + Glutamate	96.17±4.93
EtOH + Cysteine	96.63±1.42
EtOH + Pyruvate	96.75±2.82
EtOH + Aspartate	88.44±3.12[a]
EtOH + Aspartate + Pyruvate	89.55±0.49[a]

The amounts of ethanol in the effluents in response to amino acid infusion were expressed as percentages compared to those of 0.1% ethanol-perfused livers. a: $P<0.05$ with respect to EtOH group

Conversion of XDH into XO

The effect of aspartate on the ethanol-induced conversion of XDH to XO is shown in Table 3. The ratio of XDH to XO was 16.61±0.94 in control group compared to 7.89±0.37 in the ethanol-perfused group. Marked increase in the ratio of XDH to XO was observed in the liver perfused with amino acids compared to that in the single ethanol-perfused liver.

DISCUSSION

The rate of reoxidation of cytosolic NADH has been suggested as a limiting factor in ethanol oxidation (Nordmann, *et al.*, 1975: Cederbaum *et al.* 1977). In addition, the increased cytosolic NADH is involved in ethanol-induced oxidative stress. One major mechanism of ethanol-induced free radical production is suggested to be through the intracellular conversion of XDH to XO by increased ratio of NADH to NAD which would cause the enzyme to prefer oxygen to NAD, as an oxidant (Corte *et al.*, 1970: Kato *et al.* 1990: Younes *et al.* 1987). As a result of the enzymic conversion, the active enzyme would produce superoxide resulting in the increased level of ox-

Table 2. Levels of lipid peroxidation and protein carbonylation in homogenates prepared from the perfused-rat livers.

	TBARS (nmol/mg protein)	Protein Carbonyls (nmol/mg protein)
Control	0.0349±0.0020	2.35±0.57
EtOH	0.0926±0.0026	4.97±0.20
EtOH + Glutamate	0.0836±0.0020	3.18±1.04[a]
EtOH + Cysteine	0.0762±0.0096	2.98±0.28[a]
EtOH + Pyruvate	0.0556±0.0066[a]	3.21±0.84[a]
EtOH + Aspartate	0.0554±0.0040[a]	2.98±0.16[a]
EtOH + Aspartate + Pyruvate	0.0562±0.0040[a]	3.30±0.58[a]

The liver perfusd with buffer only for 40 min served as the control. Ethanol or in combination with several amino acids were infused into rat liver. a: $P<0.05$ with respect to EtOH group

idative stress.

The efficiency of NADH reoxidation depends mainly on the activity of malate-aspartate shuttle (Nordmann *et al.*, 1975: Cederbaum *et al.* 1977). This fact was illustrated in our previous study where the level of oxidative stress in ethanol perfused-rat livers was significantly modulated by infusion of aspartate, one of the direct substrate for the shuttle (Park *et al.*, 1995). The rate of ethanol oxidation was increased by about 10% compared to that of ethanol-perfused liver without aspartate supplementation. It is reasonable to speculate that aspartate supplementation would activate NAD recycling by augmentation of the shuttle, thereby its supplementation may have the desirable effects on both ethanol oxidation and ethanol-induced oxidative stress. However, it has not been compared whether other components involved directly or indirectly in the shuttle would be effective in ethanol oxidation and ethanol-induced oxidative stress as aspartate. Our previous data showed that among various amino acids tested *in vitro*, aspartate, glutamate and cysteine were effective against ethanol toxicity (Park *et al.*, 1994). However, their biochemical roles in ethanol metabolism and toxicity would be different from those of aspartate.

In the present study, the rate of ethanol oxidation was shown to be increased by about 10% in the perfused livers by aspartate infusion in combination with ethanol compared to that of the control, but infusion of glutamate, cysteine and pyruvate did not affect the metabolic turnover of ethanol significantly (Table 1). In order to identify whether there is a synergistic effect of pyruvate to aspartate on ethanol oxidation, pyruvate in combination with aspartate was simultaneously infused, since pyruvate is the direct substrate either for NAD regeneration through lactate dehydrogenase (LDH) or for α-ketoglutarate utilization by alanine aminotransferase (AAT) (Fig. 1). However, no synergistic effect of pyruvate was observed on the rate of ethanol turnover.

Though glutamate, cysteine and pyruvate did not affect the rate of ethanol oxidation significantly, they showed some effect on both ethanol-induced oxidative stress and the ratio of XDH to XO (Table 2, 3). Aspartate infusion in ethanol-perfused liver showed the significant reduction in TBARS level compared to that in single ethanol-perfused livers. Furthermore, aspartate can reduce ethanol-induced conversion of XDH into XO by modulating the cytosolic NADH/NAD ratio. However, the numeric values shown in each experiment were slightly different from the previous data (Park *et al.*, 1995). This discrepancy is mainly due to shorter time (40 min) of perfusion in this experiment. The reason for shortening the perfusion time is based on the fact that the sudden leakage of LDH into vascular effluents was observed around 50 min after perfusion. Glutamate and cysteine also showed the reduced level of protein carbonylation. This result was in accordance with that of the conversion ratio of XDH to XO (Table 3). The minute but sig-

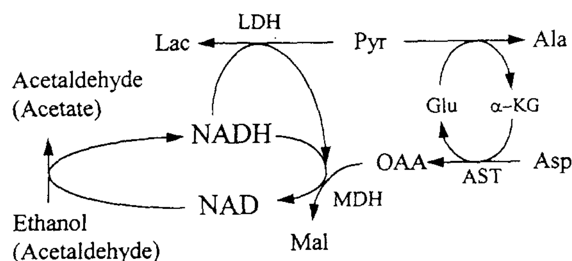

Fig. 1. Cytosolic NAD regenerating systems. LDH, lactate dehydrogenase; MDH, malate dehydrogenase; AST, aspartate aminotransferase; Lac, lactate; Pyr, pyruvate; OAA, oxaloacetate; Mal, malate; Ala, alanine; Asp, aspartate.

Table 3. Comparision of activities of XDH and XO in the perfused-rat liver tissues

	Specific activity		XDH/XO
	XDH	XO	Ratio
Control	29.52±2.95	1.78±0.08	16.61±0.94
EtOH	19.38±2.23	2.45±0.17	7.89±0.37
EtOH + Glutamate	22.53±2.25	1.82±0.06	12.40±0.81[a]
EtOH + Cysteine	20.29±1.73	1.47±0.13	13.80±0.41[a]
EtOH + Pyruvate	19.80±2.21	1.79±0.26	11.12±0.39[a]
EtOH + Aspartate	21.27±4.23	1.62±0.08	13.16±2.06[a]
EtOH + Aspartate + Pyruvate	23.91±2.67	1.60±0.06	14.49±1.62[a]

One unit (U) of both enzyme required to produce 1 μmol of NADH or 1 μmol of uric acid per min, respectively. Specific activity was calculated as miliunit (mU) per mg protein. a: $P<0.05$ with respect to etoh group.

nificant effect of glutamate on ethanol-induced oxidative stress might be explained by its immediate turnover into aspartate because of the equilibrium nature of transamination reaction. Although cysteine may not affect the metabolic turnover of ethanol (Table 1), the amino acid can scavenge acetaldehyde directly which is another source of radical generation (Cederbaum *et al.*, 1976). Therefore, the reduced level of TBARS and protein carbonylation by cysteine can be explained partially (Table 2). In case of pyruvate, it can be the direct substrate for LDH, resulting in regeneration of NAD from NADH. Thereby, pyruvate infusion may decrease the ethanol-induced conversion of XDH and reduced oxidative stress (Table 2, 3). These data are consistent with our previous results (Park *et al.*, 1994), where the cellular protective effects of amino acids against ethanol-induced toxicity were reported.

In summary, our data indicate that aspartate is the most efficient as a metabolic regulator for both metabolic turnover of ethanol and ethanol-induced oxidative stress among amino acids and organic acid used in our perfusion system. These effects of aspartate would result from NAD recycling by its supplementation through the coupled AST/MDH reactions and the malate-aspartate shuttle.

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흰쥐 간 관류 실험에서 아미노산이 에탄올 대사 및 에탄올 유래 프리라디칼의 생성에 미치는 영향

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적 요

지속적인 에탄올 산화는 세포질내 **NADH/NAD** 비의 변환을 유발한다. 이는 에탄올 대사를 제한할 뿐만 아니라 **XDH**의 **XO**로의 변환으로 프리라디칼을 생성하게 된다. 본 실험에서는 **aspartate/malate shuttle**을 효율적으로 이용하여 **NADH/NAD**의 비를 보정함으로서 에탄올 대사및 프리라디칼에 의한 **oxidative stress**를 조절할 수 있는가를 구명하였다. **Shuttle**에 관여하는 여러 아미노산과 유기산을 *in situ* **perfused liver**에 투여하여, 이들이 에탄올의 대사와 그로 인해 초래되는 산화성 세포 손상에 미치는 정도를 비교하여, 이중, **aspartate**가 가장 효율적으로 에탄올 산화를 촉진하였고 **oxidative stress**의 정도를 유의하게 낮추었음을 관찰하였다. **Glutamate, cysteine**과 **pyruvate**는 에탄올 산화에 영향이 없었지만 에탄올이 유도하는 **oxidative stress**의 정도를 감소시켰다. 이상의 결과는 아미노산을 이용하여 에탄올 산화와 에탄올 유래 산화성 손상을 대사적으로 제어할 수 있음을 보였다.