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Effects of Alanine and Glutamine on Alcohol Oxidation and Urea Nitrogen Production in Perfused Rat Liver

Jungeun Yim, Jonghee Chyun[§] and Youngnam Cha[1]

Department of Food and Nutrition, Inha University, Inchon 402-751, Korea

[1]Department of Pharmacology, School of Medicine, Inha University, Inchon 402-751, Korea

Most of the ethyl alcohol consumed by humans is oxidized to acetaldehyde in the liver by the cytoplasmic alcohol dehydrogenase (ADH) system. For this ADH-catalyzed oxidation of alcohol, NAD^+ is required as the coenzyme and NAD^+ becomes reduced to NADH. As the NAD^+ becomes depleted and NADH accumulates, alcohol oxidation is reduced. For continued alcohol oxidation, the accumulated NADH must be quickly reoxidized to NAD^+, and it is this reoxidation of NADH to NAD^+ that is known to be the rate-limiting step in the overall oxidation rate of alcohol. The reoxidation of NADH to NAD^+ is catalyzed by lactate dehydrogenase in the cytoplasm of hepatocytes, with pyruvate being utilized as the substrate.

The pyruvate may be supplied from alanine as a result of amino acid metabolism via the urea cycle. Also, glutamine is thought to help with the supply of pyruvate indirectly, and to activate the urea cycle by producing NH_3. Thus, in the present study, we have examined the effects of alanine and glutamine on the alcohol oxidation rate. We utilized isolated perfused liver tissue in a system where media containing alanine and glutamine was circulated. Our results showed that when alanine (5.0mM) was added to the glucose-free infusion media, the alcohol oxidation rate was increased by 130%. Furthermore, when both glutamine and alanine were added together to the infusion media, the alcohol oxidation rate increased by as much as 190%, and the rate of urea nitrogen production increased by up to 200%. The addition of glutamine (5.0mM) alone to the infusion media did not accelerate the alcohol oxidation rate. The increases in the rates of alcohol oxidation and urea nitrogen production through the addition of alanine and glutamine indicate that these amino acids have contributed to the enhanced supply of pyruvate through the urea cycle.

Based on these results, it is concluded that the dietary supplementation of alanine and glutamine could contribute to increased alcohol detoxification through the urea cycle, by enhancing the supply of pyruvate and NAD^+ to ensure accelerated rates of alcohol oxidation.

Key words : alcohol oxidation, alanine, glutamine, urea nitrogen

INTRODUCTION

Alcohol may be the most widely abused chemical substance in the world today. The predominant acute effects of alcohol are believed to be reduced brain function and alteration of energy metabolism in hepatic cells. Alcohol has been demonstrated to be hepatotoxic, leading to a fatty liver and cirrhosis.[1),2)]

Chronic excessive alcohol intake may lead to serious nutritional imbalance. Alcoholic subjects can replace more than 50% of their total caloric consumption by empty calories. Furthermore, alcohol interferes with the digestion, absorption, and metabolism of several nutrients.[3)]

Alcohol requires no digestion and is readily transported intact across cell membranes via passive diffusion. When administered orally, alcohol is readily absorbed from the stomach and small intestine and is distributed quickly throughout the body fluids. Small amounts of alcohol can be exhaled, unchanged, in the breath or excreted in urine and sweat, but more than 90% of absorbed alcohol is metabolized by the liver.[4)]

The identification of a method for quickly and safely lowering blood alcohol levels following a given intake could provide a mechanism for reducing alcohol's toxic effects, and could have additional benefits in the fields of alcoholism and alcohol research. In particular, providing the means for rapidly reducing blood alcohol concentrations might be useful in the treatment of acute alcohol overdose.

The factors regulating alcohol metabolism have different effects at different alcohol concentrations and under different nutritional conditions. The rate of alcohol metabolism might be affected by several factors : [5)-7)] the activities of enzyme systems oxidizing alcohol to

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[§] To whom correspondence should be adressed.

acetaldehyde, the activities of systems transporting reducing equivalents into the mitochondria, and the ability of mitochondria to oxidize these reducing equivalents. Especially, the activity of alcohol dehydrogenase (ADH),[8] located in the cytosolic fraction of the hepatocytes and which oxidizes alcohol to acetaldehyde, is a major element of alcohol metabolism. For this ADH-catalyzed oxidation of alcohol, NAD^+ is required as the coenzyme and NAD^+ becomes reduced to NADH.[9)-11)] As the NAD^+ becomes depleted and NADH is accumulated, alcohol oxidation slows and eventually stops. For continued alcohol oxidation, the accumulated $NADH^+$ must be quickly reoxidized to NAD^+, and this reoxidation of NADH to NAD^+ is known to be the rate limiting step in the overall oxidation rate of alcohol. Thus, alcohol metabolism is determined by the re-oxidation of NADH : that is, the regeneration of NAD^+.

In order to accelerate alcohol metabolism, many methods have been proposed to facilitate the regeneration of NAD^+.[12)-14)] In the cytoplasm of hepatocytes, the reoxidation of NADH to NAD^+ is catalyzed by the lactate dehydrogenase, and pyruvate is utilized as the substrate.[15),16)] The conversion of pyruvate to lactate is a reduction process, which is coupled with alcohol oxidation. Pyruvate is reduced to lactate with the help of the coenzyme NADH.

The pyruvate may be supplied from alanine, the most important of the gluconeogenic amino acids. It has been suggested that alanine is converted rapidly to pyruvate by the transamination process, following which the pyruvate is reduced to lactate, thereby reoxidizing NADH. In this pathway, ammonia is generated, and is converted into urea. Glutamine is thought to help with the supply of pyruvate indirectly, and to activate the urea cycle by producing NH_3.

Thus, in the present study, we examined the effects of alanine and glutamine on the alcohol oxidation rate and urea nitrogen production; if these effects enhance alcohol metabolism, then alcohol-induced hepatotoxicity may be reduced. We utilized an isolated perfused liver system[17] which is recirculated with media containing alanine and glutamine; this method has been found useful elsewhere in studying nutrient metabolism.

METHOD

1. The Animals

Male Sprague Dawley rats, weighing 200 to 250gm, were used in the experiment. Prior to the experiment, animals were housed for at least 3 or 4 days in the laboratory animal facility in polypropylene cages. The lighting in the animal room was regulated by an automatic control switch such that lights were on from 7 a.m. to 7 p.m. and off from 7 p.m. to 7 a.m. The rats were fed a pelleted diet of Samyang Company chow and were given water *ad libitum*. All animals were fasted for 24 hours prior to the experiment, to ensure glycogen depletion.

2. The Recirculating liver perfusion system[17]

Rats were anesthetized by an intraperitoneal injection of sodium secobarbital (40mg/kg body weight). Livers were perfused using a recycle method with a modified Krebs-Henseleit bicarbonate buffer (KHB) (Table 1); livers were equilibrated with oxygen and carbon dioxide (95 : 5) at 37℃ and infused at a flow rate of about 3~4 ml per minute per gram of liver (Figure 1). Perfusion with KHB was maintained for 5 minutes for equilibration. Subsequently, the perfusion medium was switched to the KHB solution containing alcohol (0.02%, v/v) and perfusion was continued for 30 or 60 minutes by adding alanine and glutamine (Figure 2). Perfusates were collected at 5 minute intervals, and the collection time was 20 seconds.

Table 1. Composition of Krebs-Henseleit bicarbonate buffer

Ingredien	Final Buffe		Stock Solutio		
	mmole/	g/	mol	g/	ml/
NaCl	118.00	6.90	2.36	137.9	50
KCl	4.70	0.35	0.47	35.0	10
$CaCl_2$	2.52	0.29	0.25	27.0	10
$MgSO_4 \cdot 7H_2O$	1.64	0.14	0.16	40.4	10
KH_2PO_4	1.18	0.16	0.12	16.1	10
$NaHCO_3$	24.88	2.09			

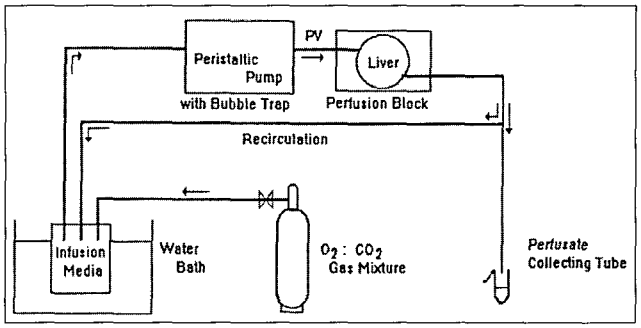

Fig 1. Diagram for recirculating isolated perfused liver

3. Alcohol concentrations in perfusates[18]

The alcohol concentrations of perfusates were analyzed by a modified method of Buchner and Redetzki; 2.7mL of 0.5M glycine-NaOH buffer (pH 9.0) was placed in a test tube, 100uL of 25mM NAD^+ was added, and then 100ul of the perfusates and 100ul of 50 unit alcohol dehydrogenase were then vigorously mixed by a vortex mixer. After 10 minutes, the absorbance was read at 340 nm.

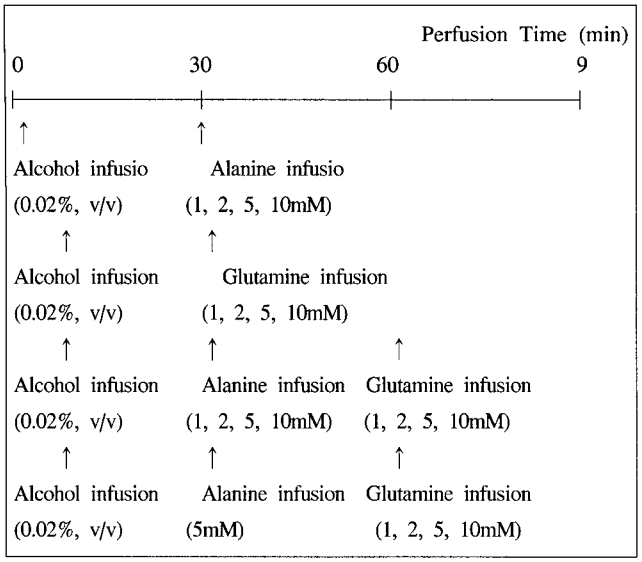

Fig 2. Experimental Design

4. Urea nitrogen concentrations in perfusates[19]

The urea nitrogen concentrations of perfustates were analyzed by the Oxime method of Coulombe and Favreau. 1.4ml of sulfuric acid, phosphoric acid, and ferric acid were placed in a test tube for hydrolysis of 2,3-butanedione monoxime; 1.4mL of oxime solution and 200uL of the collected perfusates were then added. The product was vigorously mixed by a vortex mixer, boiled in water for 20 minutes, and then set aside to attain room temperature. Finally, the absorbance was read at 520 nm.

5. Calculation of perfusate alcohol parameters and statistical analysis.

In order to describe the time course of the perfusate alcohol curve in an individual animal following a dose of alcohol, the one-compartment open model with zero-order elimination was used. The slope of the elimination phase was calculated by a linear least squares regression of the descending linear portion of the perfusate alcohol curve. The means and standard deviations were calculated for all treatment groups. The data were subjected to analysis of variance followed by One-Way ANOVA tests to determine which means were significantly different from each other. In all cases a p-value of ≤ 0.05 was used to determine significance.

RESULTS

1. The effects of various concentrations of alanine on the alcohol oxidation rate and on the activation of the urea cycle.

To observe the influence of alanine during alcohol metabolism, we utilized the recirculating perfused liver system. First, the decrease in alcohol concentration with time was observed 30 minutes after perfusion using only alcohol; the alcohol oxidation rate was found to be 137.00±22.50 nmoles/min (0-30 minutes). Subsequently, the change in alcohol metabolism with time was observed following the infusion of alanine. Various concentrations (0, 1, 2, 5, and 10mM) of alanine were infused from 30 to 90 minutes after perfusion. The alcohol oxidation rate without alanine was 78.78±12.12 nmoles/min, while equivalent rates for infusions of alanine at levels of 5mM and 10mM were 103.89±15.83 nmoles/min and 100.47±17.40 nmoles/min, respectively. This shows that the alcohol oxidation rate has significantly increased by 5mM and 10mM infusions of alanine, compared to the samples without alanine ($p<0.05$) (Table 2).

To observe the effects of alanine on urea cycle activity, alanine was infused at various concentrations (0, 1, 2, 5, and 10mM), and the subsequent production of urea nitrogen was measured. The results showed that the production of urea nitrogen increased somewhat with increased levels of alanine infused, but this was not significant (Table 2); that is, the infusion of alanine alone contributed to significant increases in alcohol metabolism, but insignificant increases in urea cycle activity.

Table 2. Effect of alanine or glutamine to the perfusion media on alcohol oxidation rate and urea nitrogen production rate.

		Alcohol oxidation rate (nmoles/min)	Urea nitrogen production rate (nmoles/min)
Ala[a]	0 mM	78.78 ± 12.12	40.53 ± 6.17
	1 mM	89.05 ± 10.25	50.99 ± 8.11
	2 mM	97.04 ± 10.90	56.33 ± 7.43
	5 mM	103.89 ± 15.83*	65.13 ± 8.87
	10 mM	100.47 ± 17.40*	61.34 ± 5.23
Gln[b]	0 mM	78.78 ± 12.12	40.53 ± 6.17
	1 mM	81.67 ± 15.21	60.20 ± 7.50
	2 mM	85.00 ± 15.43	66.86 ± 12.12
	5 mM	80.00 ± 16.32	70.20 ± 18.85*
	10 mM	76.67 ± 12.21	70.20 ± 10.53*

a Ala : Alanine
b Gln : Glutamine
c Values represent the mean±S.D. of four replicates
* $P<0.05$ versus untreated control group

2. The effects of various concentrations of glutamine on alcohol oxidation rates and on urea cycle activity

The effects of various concentrations of glutamine on alcohol metabolism are depicted in Table 2. With increases in the levels of glutamine, the alcohol oxidation rate increased; the samples with 2mM glutamine achieved an alcohol oxidation rate of 85.00±15.43 nmoles/min compared to 78.78±12.12 nmoles/min for the samples

without glutamine, but this difference was not significant.

To observe the effects of differing levels of glutamine on urea cycle activity, glutamine was infused at various concentrations (0, 1, 2, 5, and 10mM), and the production of urea nitrogen was measured. The results showed that the production of urea nitrogen significantly increased to 70.20±18.85 nmoles/min and 70.52±10.53 nmoles/min for the groups with 5 mM and 10 mM glutamine infusions, respectively ($p<0.05$). That is, the infusion of glutamine alone did not contribute to significant increases in alcohol metabolism, but significantly increased urea cycle activity.

3. The combined effects of various concentrations of alanine and glutamine on alcohol metabolism and on urea cycle activity

To observe the effects of simultaneous infusions of alanine and glutamine on alcohol metabolism and urea cycle activation, both alanine and glutamine were simultaneously injected in proportional concentrations (Table 3). With the simultaneous infusions of an alanine/glutamine combination at levels of 5 : 5mM and 10 : 10mM, alcohol metabolism significantly increased to 146.89±10.72 nmoles/min and 126.5±10.6 nmoles/min ($p<0.05$), respectively. The increases in alcohol metabolism induced by simultaneous infusion were much larger than when alanine alone was infused. Especially, the effect of the 5 : 5mM infusion was the most effective in increasing the alcohol oxidation rate.

The simultaneous infusion of alanine and glutamine was also effective in increasing urea cycle activity. Among the various results, the injections of 2 : 2mM, 5 : 5mM, and 10 : 10mM of alanine/glutamine resulted in significant increases in urea nitrogen production of 75.20±12.66, 79.27±9.78, and 79.36±14.52 nmoles/min, respectively ($p<0.05$) (Table 3). The increases in urea nitrogen production as a result of simultaneous infusion (at levels of 5 : 5mM and 10 : 10mM) were larger than when glutmaine alone was used.

4. The effects of differing ratios of alanine to glutamine on the alcohol oxidation rate and on urea cycle activity

To determine the most effective combination of alanine and glutamine for increasing alcohol oxidation rates and urea cycle activity, different alanine/glutamine ratios were infused (Table 3). The 1 : 1 combination of 5 : 5mM resulted in the highest rates of alcohol metabolism (146.89±10.72 nmoles/min) and of urea nitrogen production (79.27±9.78 nmoles/min); these increases are 1.4 times the level of alcohol metabolism and 1.2 times the level of urea nitrogen production compared to the case of a 5mM infusion of alanine alone. In the case of the 1 : 1 alanine/glutamine infusion, alcohol metabolism increased by 1.9 times and urea nitrogen production increased by 2 times, compared to samples without any infusion.

Table 3. Effects of adding alanine and glutamine to the perfusion media on alcohol oxidation rate and urea nitrogen rate.

		Alcohol oxidation rate (nmoles/min)	Urea nitrogen production rate (nmoles/min)
Ala[a] : Gln[b] (by amount)	0:0 mM	78.78 ± 12.12	40.53 ± 6.17
	1:1 mM	93.17 ± 8.93	56.86 ± 10.10
	2:2 mM	114.83 ± 20.50	75.20 ± 12.66*
	5:5 mM	146.89 ± 10.72*	79.27 ± 9.78*
	10:10 mM	126.50 ± 10.60*	79.36 ± 14.54*
Ala : Gln (by ratio)	0:0 mM	78.78 ± 12.12	40.53 ± 6.17
	5:0 mM	103.89 ± 15.83	65.13 ± 8.87
	5:1 mM	115.97 ± 16.21	65.13 ± 10.21
	5:2 mM	121.02 ± 20.55	73.81 ± 9.77
	5:5 mM	146.89 ± 10.72*	79.27 ± 9.78*
	5:10 mM	128.68 ± 22.77	77.27 ± 10.11*

a Ala : Alanine
b Gln : Glutamine
c Values represent the mean±S.D. of four replicates
* $P<0.05$ versus untreated control group

DISCUSSION

Alcohol is oxidized to acetaldehyde via NAD^+, the coenzyme of Acohol Dehydrogenase, and an increased $NADH/NAD^+$ ratio results in a corresponding increase in the lactate/pyruvate ratio. It is necessary to regenerate NAD^+ to further oxidize the alcohol load, indicating that oxidizing agents such as pyruvate would be necessary to convert NADH back to NAD^+.

It was reported that the rates of reduction of acetaldehyde and pyruvate were correlated with the concentration of these metabolites and NADH, and the highest rate was observed for fructose metabolism in the fed state. It was suggested that the dissociation of enzyme-bound NAD^+ as well as NADH may be rate-limiting steps in the alcohol dehydrogenase reaction.[20] Also, another study found that the regeneration of NADH in the cytosol is limited in chicken liver, and that gluconeogensis is regulated in part by alteration in the redox states of mitochondria and cytosol.[21]

The results of the present study show that when alanine (5.0mM) was added to the infusion media, the alcohol oxidation rate was increased by 1.3 fold. It is possible that alanine could serve as a source of pyruvate by undergoing oxidative deamination to ketoacids. Another study has suggested that with an adequate supply of alanine as a substrate, ethanol stimulates gluconeogensis in normal rat liver but has no affect on already enhanced

gluconeogensis in thyrotoxic rat liver.[22] L-Alanine is reported to supply an abundance of the pyruvic acid that is involved in the NAD^+-generating system.[23]

Our results showed that the addition of glutamine (5.0mM) alone to the infusion media did not accelerate alcohol oxidation. When the glutamine was added together with alanine, the alcohol oxidation rate was increased by up to 1.9 fold and the rate of urea nitrogen production was increased by up to 2 fold. Glutamine apparently contributes to enhancing the supply of pyruvate and NAD^+ for accelerated alcohol oxidation rate, and to enhancing the urea cycle's ability to detoxify alcohol.[24),25]

The pyruvate from alanine is converted to lactate, and regenerates NAD^+ which is essential for the ADH reaction. Glutamine supplementation accelerates the coupling reaction of alcohol-acetaldehyde and pyruvate-lactate by eliminating NH_3, thereby activating the urea cycle. Glutamine is converted to glutamate by deamination, and glutamate, in turn, will be converted to alpha-ketoglutarate. Glutamine also appears to enhance the urea cycle by assisting with the synthesis of carbamoyl phosphate. Carbamoyl phosphate synthetase is inactive in the absence of its allosteric activator, N-acetylglutamate. This activator is synthesized from acetyl CoA and glutamate by a liver enzyme.

Our data demonstrated that the lactate dehydrogenase system (lactate-pyruvate) gave reliable values for the cytosolic NAD^+/NADH ratio. Alcohol-induced changes in cytosolic NAD^+/NADH ratios from the lactate/pyruvate system were prevented by treatment with alanine and glutamine; the strength of the effects of this treatment depended on the concentrations of and the ratios of the alanine/glutamine infusions.

It is possible that alanine improves alcohol metabolism through increased supplies of pyruvate, and that glutamine accelerates alcohol metabolism by activating the urea cycle for alcohol detoxification.

Finally, our study demonstrates that the isolated perfused liver system methodology, generally employed to assess changes in hepatic drug metabolism using a number of model drug substrates, can be effectively adapted for the study of nutrient metabolism.

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