CONTRIBUTION OF "SUBSTRATE SHUTTLES" IN HEPATIC ALCOHOL METABOLISM IN CHRONIC ALCOHOLISM*

Arun K. Rawat and K. Kuriyama

Division of Biochemistry and Neuropharmacology, Department of Psychiatry, Downstate Medical Center, State University of New York

Brooklyn, New York 11203

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Summary: Chronic alcohol-feeding to mice results in an increased utilization of DPNH by hepatic mitochondria, in the presence and absence of "malate-aspartate shuttle" components. The utilization of alcohol and α-glycerophosphate by these mitochondria is also increased. Succinic dehydrogenase activity assayed with phenazine methosulphate as an index of mitochondrial permeability showed higher activity in mitochondria from chronic alcohol-treated mice compared to pair-fed controls. Alcohol-withdrawal from chronically-treated animals showed a lack of correlation between blood alcohol clearance, and hepatic microsomal alcohol-oxidizing activity on one hand, and between blood alcohol clearance and alcohol dehydrogenase activity on the other.

Introduction: It has been observed by several workers that mitochondria isolated from normal liver have a rather low permeability to nicotinamide adenine dinucleotides (1,2). During hepatic metabolism of alcohol by alcohol dehydrogenase, two molecules of DPNH are generated per molecule of alcohol oxidized. It has been suggested that the reoxidation of this DPNH may be a rate-limiting factor in the metabolism of alcohol (3,4). Although several "substrate shuttles" have been proposed for the transport of extra-mitochondrial DPNH across the mitochondrial membranes (5,6). Their contribution in normal liver is rather limited (7-9). However, the contribution of these shuttles in livers from chronically alcohol-treated animals in the transport of extramitochondrial DPNH has not been evaluated. Prolonged alcohol-treatment causes hepatic

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injury by affecting cellular and mitochondrial membranes as shown by the electron microscopic studies (10). Livers from alcoholics and chronic alcohol-treated animals show changes in the ultrastructure of mitochondria along with increased mitochondrial fragility and lysis of mitochondrial membrane (11,12).

The present study was undertaken to investigate (a) the biochemical consequences of morphological changes produced by chronic alcohol intake in hepatic mitochondria, (b) alteration in the contribution of "substrate shuttles" in the transport of extramitochondrial DPNH into the mitochondria and (c) influence of altered mitochondrial permeability on the rate of alcohol utilization.

Experimental: Male albino mice weighing 25-28 g were pair-fed on liquid diet for four weeks. The liquid diet consisted of 61% commercial Metrecal, 6% (w/v) ethyl alcohol or isocalorid sucrose. Alcohol was withheld in all cases 20 hrs. before sacrifice, unless otherwise stated. Animals were sacrificed by decapitation, and their livers homogenized in 0.25 M sucrose. Mitochondria were prepared according to Hogebloom (13). Microsomes and supernatant fractions were obtained by further centrifugation at 105,000 Xg for 60 minutes. Washed preparations of organelles were used for biochemical studies.

Studies with isolated mitochondria: The rates of extramitochondrial DPNH utilization were studied by mitochondria from chronic alcohol-treated and control animals in the presence or absence of "malate-aspartate shuttle" components. Mitochondria were incubated in an oxygenated medium containing 20 mM KH_2PO_4, 20 mM Tris-HCl, 5 mM $MgCl_2$ (pH 7.35), 5 mM glutamate, 1 mM malate, 2 mM aspartate, and 25 uM DPNH. In experiments where full component of the "shuttle" were present, malate dehydrogenase (7 units/ml), and glutamate-oxaloacetate transaminase (7 units/ml) were also added. In state 3 respiration ADP (1 mM) was added. The rates of DPNH disappearance were measured fluorometrically. For studying alcohol utilization by the mitochondria

in a reconstituted system, ethanol (10 mM) and dialysed yeast alcohol dehydrogenase (3 units/ml) were present additionally. Samples were taken at 2 minute intervals for the assay of ethanol in the medium. Mitochondrial succinic dehydrogenase activity using phenazine methosulphate (14), and α-glycerophosphate utilization were determined manometrically. Protein was determined colorimetrically

Studies with isolated microsomes: The microsomal pellet obtained after 105,000 xg centrifugation was used for studies of microsomal ethanol oxidation. The reaction mixture contained 100 µ moles of ethanol, 5.0 µ moles of TPNH and 5 mg of microsomal protein in a final volume of 1 ml. The stoppered tubes (15) were incubated for 30 min. at 37°C. Incubation medium was removed every 10 min., deproteinized with $HClO_4$ and remaining ethanol determined enzymatically (16).

The rates of alcohol disappearance from the blood: Ethanol disappearance from the blood was determined in all animals over a period of 4 hrs. Alcohol (3 g/kg) was given as a 20% aqueous solution intraperitoneally. After an initial equilibriation period of 45 min., blood samples (50 µl) were obtained hourly and alcohol determined enzymatically. Alcohol concentration in blood when plotted against time followed a linear function. The rates of alcohol disappearance from the blood were calculated from the slope of this line.

Results and discussion: In the present investigation we have shown that the mitochondria isolated from livers of animals given alcohol for 4 weeks (alcohol withheld 20 hrs. before sacrifice) utilized externally added DPNH, ethanol and α-glycerophosphate at significantly higher rates than the corresponding controls. (Table 1). Succinic dehydrogenase-phenazine methosulphate activity assayed as a measure of the mitochondrial permeability also showed higher activity in livers from chronic alcohol-treated animals compared to pair-fed controls (Table 1). In chronic alcohol-fed animals the permeability of

TABLE 1.

Effect of chronic alcohol-administration on the rates of DPNH, ethanol, and α-glycerophosphate oxidation and the activity of succinic dehydrogenase by hepatic mitochondria.

Treatment	Rate of DPNH oxidation			Ethanol oxidation	α-glycerophosphate oxidation	Succinic dehydrogenase activity
	no shuttle (state 4)	with shuttle (state 4)	with shuttle (state 3)	with shuttle (state 3)		
	(n moles/mg protein/min.)			(n moles/mg protein/min.)	(μl O_2/mg protein/10 min.)	(μl O_2/mg protein/10 min.)
Control	13.3 ± 1.2	24.6 ± 1.9	40.3 ± 3.5 (6)	28.9 ± 2.0 (6)	2.8 ± 0.2 (6)	12.0 ± 1.1 (6)
Alcohol	18.8 ± 1.5	31.0 ± 2.7	56.9 ± 4.1 (6)	36.5 ± 3.0 (6)	5.9 ± 0.4 (6)	18.8 ± 1.9 (6)

The experimental details are given in the text. The results are expressed as average ± SEM.
The number of experiments are given in parenthesis, livers from 6 animals were pooled in each experiment.

mitochondrial membranes to DPNH is no more a rate-limiting factor in the metabolism of alcohol. The contribution of "malate-aspartate shuttle" in the transport of extra-mitochondrial DPNH into the mitochondrial compartment is increased in chronic alcohol-fed group (Table 1). This finding is compatible with the view that ethanol feeding increases the mitochondrial permeability rendering the substrates more accessible to the intra-mitochondrial enzyme sites and the respiratory chain. In view of the fact that the mitochondrial membranes are rich in lipids, it is not surprising that prolonged alcohol treatment affects the properties of these membranes. After 4 weeks of chronic alcohol-treatment the rates of blood alcohol clearance were highest (Table 2). Alcohol withdrawal experiments show that there was, however, a lack of correlation between the rates of

TABLE 2.

Effect of alcohol withdrawal on the rate of blood ethanol clearance, hepatic alcohol dehydrogenase, and TPNH dependent ethanol oxidation in chronic alcohol-treated mice.

Days after withdrawal	Blood alcohol clearance (mg./ 100 ml hr.)		Alcohol dehydrogenase activity (n moles/mg. supernatant protein/min.)		TPNH dependent alcohol oxidation (n moles/mg. microsomal protein/min.)
0	30.4 ± 3.0	(6)	10.1 ± 1.0	(6)	8.1 ± 0.9
10	22.6 ± 2.1	(6)	17.0 ± 1.5	(6)	5.9 ± 0.7
20	20.4 ± 2.0	(6)	11.1 ± 1.0	(6)	7.8 ± 0.8
30	20.0 ± 2.0	(6)	8.0 ± 0.9	(6)	8.0 ± 0.9

The experimantal details are given in the text. The results are expressed as average ± SEM. The number of experiments are given in parenthesis, four animals were used in each experiment.

blood alcohol clearance and microsomal ethanol oxidizing activity on one hand, and between blood alcohol clearance and alcohol dehydrogenase

activity on the other (Table 2).

The original findings of Orme-Johnson and Ziegler (17), demonstrated the capability of hepatic microsomes to metabolize alcohol. Hereto, it has been hypothesized (18) that the microsomal system may account for the increased rate of alcohol metabolism observed in the chronically alcohol-treated animals. However, several investigators question this hypothesis since experimental evidence for this claim has not been presented.

It has been suggested that the capacity of microsomes to metabolize ethanol is in all probability due to the unspecificity of the system with questionable physiological significance (19). Our observations (Table 2) also show a lack of correlation between the rates of blood alcohol clearance and microsomal ethanol-oxidizing system. Chronic phenobarbital-treatment of the rats which also induces the microsomal ethanol-oxidizing activity, failed to increase the rate of alcohol metabolism in these animals (20).

Observations recorded in this report show that the low permeability of the mitochondrial membranes to extra-mitochondrial DPNH observed in livers from normal animals is altered upon chronic alcohol-feeding. The permeability of the mitochondria to DPNH and other substrates is increased in chronically alcohol-treated animals, and is no more a rate-limiting factor in the metabolism of alcohol by these mitochondria. The increased alcohol metabolism observed in chronic alcohol-treated animals is associated with increased metabolism of alcohol by the hepatic mitochondria from these animals. The morphological changes in the ultrastructure of mitochondria caused by chronic alcohol-treatment result in changes in the membrane permeability behaviour of the mitochondria, adapting itself to increased need for alcohol metabolism during prolonged alcohol consumption.

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