EFFECT OF CHRONIC ETHANOL INGESTION ON MITOCHONDRIAL PERMEABILITY AND THE TRANSPORT OF REDUCING EQUIVALENTS

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Received September 11,1972

Summary

Chronic ethanol feeding to rats leads to an increased rate of ethanol metabolism. This increase is not accompanied by altered permeability of the mitochondria to NADH. The activities of the reconstituted malate-aspartate, fatty acid elongation and α-glycerophosphate shuttles for the transport of reducing equivalents into mitochondria were not changed by chronic ethanol ingestion. Thus, in contrast to other reports, these results show that neither increased mitochondrial permeability nor enhanced shuttle activity explains the acceleration of ethanol metabolism produced by chronic ethanol ingestion.

Chronic administration of ethanol to rats accelerates the rate of ethanol metabolism (1,2), but the reasons are unclear. The oxidation of ethanol by alcohol dehydrogenase (ADH) generates reduced nicotinamide adenine dinucleotide (NADH) in the cytosol; reoxidation of NADH has been suggested as the rate-limiting factor in the overall metabolism of ethanol (3,4). To the extent that the capacity of mechanisms in the cytosol for the reoxidation of NADH is exceeded, reducing equivalents derived from NADH must be transported into the mitochondria to be oxidized by the electron transport chain (5). Since mitochondria are relatively impermeable to NADH, several shuttle mechanisms have been proposed for the transport of reducing equivalents into the mitochondria. The most important are the malate-aspartate shuttle (6), the fatty acid elongation shuttle (7) and the α-glycerophosphate shuttle (8), all of which have been reconstituted in vitro using isolated mitochondria (5,9).

In a recent study, Rawat and Kuriyama (10) reported that chronic ethanol administration to mice leads to increased utilization of NADH by mitochondria and to increased activity of

the malate-aspartate shuttle. In the present study, activities of the reconstituted malate-aspartate, fatty acid and α-glycerophosphate shuttles were compared in hepatic mitochondria from rats chronically fed ethanol or isocaloric carbohydrate.

Materials and Methods

Male Sprague-Dawley rats, initially weighing 150 gm, were fed a liquid diet in which carbohydrate provided 36 percent of total calories over a 24 day period (11). Pair-fed littermates were given the same diet except that ethanol was isocalorically substituted for carbohydrates. In seven pairs, blood ethanol clearance rates were determined after administering intragastric ethanol (3 gm per Kg) following an overnight fast. Blood samples were obtained from the tail vein every half hour for 6 1/2 hours, and the rate of ethanol clearance measured as described previously (12). In 14 pairs, the rats were decapitated, and hepatic mitochondria were prepared by methods previously described (13). To study the effects of acute administration, ethanol, 5 gm per Kg body weight, was given by stomach tube as a 50 percent (w/v) solution to three rats. Controls were given isocaloric glucose in the same volume. The rats were killed 3 hours later, and the hepatic mitochondria were isolated.

Reconstitution of the shuttles: The equilibrium of the ADH reaction favors formation of ethanol and NAD^+ from acetaldehyde and NADH. Therefore, the rate of ethanol disappearance would be quite low in the absence of a shuttle mechanism to remove one of the products of the reaction (NADH). Since dissociation and reoxidation of NADH bound to the enzyme probably represent the rate limiting step in the ADH reaction (3), the rate of ethanol disappearance in these systems reflects the rate of passage of reducing equivalents into the mitochondria (9). Mitochondria (5-10 mg protein) were suspended in a medium containing 300 mM mannitol, 10 mM phosphate buffer, pH 7.4, 10 mM tris-HCl, pH 7.4, 10 mM KCl, 5 mM $MgCl_2$, 1 mM ADP, and H_2O to a final volume of 3.0 ml. A NADH generating system was produced by the addition of 0.25 mM NAD^+, 6 mM ethanol and 16 units of ADH. The fatty acid shuttle was reconstituted by adding 1 mM ATP, 0.2 mM coenzyme A and 0.1 mM of albumin-bound

fatty acid. The malate-aspartate shuttle was assembled by adding 3.3 mM aspartate, 1.33 mM α-ketoglutarate, 3 units of malate dehydrogenase and 2 units of glutamic-oxalacetic transaminase. The α-glycerophosphate shuttle was formed by adding 1 mM dihydroxyacetone phosphate, 1 mM ATP and 1 unit of α-glycerophosphate dehydrogenase. The components of each shuttle system were added and the flasks were incubated at 30° for 2 minutes. The reaction was then initiated by the addition of ethanol. The flasks were immediately sealed and maintained at 30° in a Dubnoff metabolic shaker for 20 minutes. The reaction was terminated by the addition of TCA, aliquots were removed, and the remaining ethanol concentration was determined by the method of Bonnichsen (14). Blank flasks contained the TCA added before the ethanol. The endogenous rate, i.e., the rate of ethanol disappearance in the absence of the added shuttle components, was determined as above, except for the omission of fatty acid, α-ketoglutarate, or dihydroxyacetone phosphate.

Permeability studies: Mitochondria are freely permeable to NH_3. When the accompanying anion traverses the mitochondrial membrane, water will enter to maintain isosmotic conditions. The rate of mitochondrial swelling, measured spectrophotometrically, in the presence of ammonium salts, was therefore taken as an index of mitochondrial permeability to anions, according to the method of Chappell and Haarhoff (15).

Results

Rats chronically fed ethanol displayed a significant acceleration in the rate of blood ethanol clearance (Table 1). The endogenous rates of ethanol oxidation obtained in all 3 reconstituted shuttles was the same for mitochondria from rats chronically fed ethanol and from their pair-fed controls (Table 2). This rate is essentially a measure of external NADH penetration into the mitochondria, plus the contribution made by endogenous shuttles. Thus, there is no increased permeability toward NADH in mitochondria from ethanol treated rats. This conclusion was confirmed by polarographically assaying the ability of the mitochondria to oxidize externally added NADH; oxygen consumption was comparable in mitochondria derived from ethanol-fed and control animals. Mean control oxygen consumption was 1.65

TABLE 1

EFFECT OF ETHANOL FEEDING FOR 24 DAYS ON BLOOD ETHANOL CLEARANCE

	Blood Ethanol Clearance (mg/hour)	
	per liter	per Kg body weight
Ethanol	414 ± 54*	362 ± 47
Controls	283 ± 39**	248 ± 34

* Mean ± S.E.M.

** Differences between controls and ethanol-fed rats are statistically significant by the paired t test ($p < .01$).

TABLE 2

ACTIVITIES OF SHUTTLES FOR TRANSPORTING REDUCING EQUIVALENTS IN MITOCHONDRIA FROM ETHANOL FED RATS AND THEIR PAIR-FED CONTROLS. NO SIGNIFICANT DIFFERENCES ARE NOTED BETWEEN THE TWO GROUPS.

	n moles ethanol oxidized/min./mg mitochondrial protei	
Shuttle	Control	Ethanol
Malate-aspartate		
Endogenous	5.33 ± 0.29*	5.19 ± 0.39
Complete (α-ketoglutarate)	17.08 ± 1.57	18.46 ± 2.13
Fatty acid		
Endogenous	5.71 ± 0.32	5.68 ± 0.39
Palmitate	9.88 ± 0.77	9.73 ± 0.69
Oleate	10.78 ± 1.18	9.48 ± 0.81
Octanoate	10.84 ± 1.24	10.66 ± 0.96
α-glycerophosphate		
Endogenous	5.48 ± 0.37	5.29 ± 4.1
Complete (dihydroxyacetone phosphate)	16.5 ± 3.50	13.5 ± 1.74

* Mean ± S.E.M.

n atoms per minute per mg protein, compared to 1.58 in mitochondria from ethanol fed rats.

In vitro addition of ethanol (up to 0.4 M) had no effect on NADH penetration, measured polarographically.

Table 2 also indicates that the effectiveness of all 3 shuttles in transporting reducing equivalents into the mitochondria is unaltered by chronic ethanol consumption. Moreover, the immediate production of reducing equivalents causes no alteration in shuttle activity, as evidenced by the fact that acute ethanol intoxication had no effect on any of the 3 shuttle systems. The permeability of the mitochondria to anions, as measured by the rate of swelling when exposed to various NH_4^+ salts, was unchanged by ethanol feeding (Table 3).

Discussion

Data reported in this study indicate that hepatic mitochondria derived from ethanol-fed rats show the same permeability toward NADH as those from controls pair-fed isocaloric carbohydrate. In addition, the demonstration of unchanged permeability to various anions, particularly malate and glutamate, which are necessary for activity of the malate-aspartate shuttle, indicates that chronic ethanol feeding does not enhance the penetration of substrates which participate in this shuttle mechanism. The activities of the reconstituted malate-aspartate, fatty acid and α-glycerophosphate shuttles were comparable in the 2 groups of mitochondria. These data are at variance with those of Rawat and Kuriyama (10), who reported not only increased activity of the malate-aspartate shuttle, but also increased utilization of NADH in alcohol-fed mice. Rawat and Kuriyama found a mean endogenous NADH oxidation rate of 13.3 nmoles per mg mitochondrial protein per minute under state 4 conditions in control mitochondria, in the absence of any shuttle components. This rate is extremely high, and is about the same as that which we and others (16, 17) have found for NAD-linked oxidation of substrates, such as glutamate and β-hydroxybutyrate, by rat liver mitochondria under state 4 conditions. We doubt that this high rate of endogenous NADH oxidation can be attributed to a species difference between rats and mice, since Sactor and Dick (18) demonstrated that intact mouse liver mitochondria are impermeable to NADH. Because Rawat and Kuriyama's incubation medium was highly hypotonic, no sucrose or KCl being added to maintain isosmotic conditions, mitochondrial swelling would be expected, particularly in the presence of 20 mM phosphate. The most likely

TABLE 3

PERMEABILITY OF MITOCHONDRIA TO ANIONS AS MEASURED BY SWELLING IN ISOTONIC SOLUTIONS OF VARIOUS AMMONIUM SALTS. NO SIGNIFICANT DIFFERENCES ARE NOTED BETWEEN MITOCHONDRIA FROM CONTROL AND ETHANOL FED RATS.

	Swelling Rates (Δ O.D./5 min./mg protein)	
Anion	Control	Ethanol
phosphate	0.420	0.460
malate	0.272	0.297
glutamate	0.160	0.150
citrate	0.073	0.078

interpretation of their abnormally high rates of NADH oxidation is therefore mitochondrial damage; the differences between the two groups of mouse mitochondria were probably merely reflections of different sensitivities of the mitochondria to damage by their preparative methods. We have indeed demonstrated that mitochondria from ethanol-treated rats are more sensitive to injury in vitro (19). Thus, in any event, the observed acceleration of ethanol metabolism produced by chronic ethanol consumption cannot be explained by an increased rate of transport of reducing equivalents into the mitochondria.

Acknowledgements

These studies were supported in part by USPHS grants MH-20067, MH 15558 and AM 12511.

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