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EFFECTS OF CHRONIC ETHANOL FEEDING AND ACETALDEHYDE METABOLISM ON CALCIUM TRANSPORT BY RAT LIVER MITOCHONDRIA

Mark A. Korsten*, Ellen R. Gordon, Jeffrey Klingenstein and Charles S. Lieber

Alcohol Research Center, V.A. Medical Center, Bronx, N.Y.
and Mount Sinai School of Medicine (CUNY), N.Y. N.Y.

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The prolonged feeding of ethanol to rats alters in vitro mitochondrial transport of calcium. Hepatic mitochondria isolated from rats fed ethanol for 7 weeks exhibited decreased retention of calcium in the presence of 4mM-Pi. This defect was associated with enhanced efflux of calcium when mitochondria were incubated with EGTA. Acetaldehyde at low, "physiological" concentrations (100μM) enhanced calcium retention by mitochondria but this response was blunted after chronic ethanol administration. The in vitro actions of acetaldehyde appear to be mediated, in part, by its metabolism in mitochondria since pretreatment of rats with cyanamide (an aldehyde dehydrogenase inhibitor) prevents this effect.

Calcium transport is an important energy-dependent mitochondrial function, but little is known about the chronic effects of ethanol on this process. We studied these effects since it is increasingly apparent that mitochondria play a key role in controlling the distribution of intracellular calcium (1) and thereby the activities of a variety of calcium-sensitive enzymes (2,3). In addition, we investigated the in vitro effects of acetaldehyde since this metabolite of ethanol has been implicated in the toxic effects of chronic ethanol consumption (4). Tissue levels of acetaldehyde during ethanol oxidation are estimated to be between 100-250μM (5-7); unlike previous studies that assessed the effects of very high levels of acetaldehyde on calcium transport, we now employed a level (100μM) that is considered "physiological".

MATERIALS AND METHODS

Animals and Feeding Methods

Male Sprague Dawley rats were fed a liquid diet (Bio-Serv, Frenchtown, N.J.) for 5-7 weeks in which ethanol provided 36% of total calories. Pair-fed controls were treated in the same manner except that carbohydrate isocalorically replaced ethanol (8). In studies involving "chow"-fed rats, male littermates were fed a standard laboratory diet (Ralston Purina Co., St. Louis, MO) ad libitum. Rats were sacrificed after an overnight fast and weighed approximately 225-250 g at time of death. A number of "chow" rats were given cyanamide (5 mg/kg I.P.) 90 minutes prior to sacrifice. In cyanamide experiments, control rats were given equal volumes of isotonic (.9%) saline by I.P. injection.

*To whom correspondence should be addressed.

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Preparation of Mitochondria

Mitochondria were isolated by the method of Johnson and Lardy (9) in 10 volumes of ice-cold 0.25M sucrose, 5mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), 0.5mM EGTA (pH 7.4). The recovery of mitochondria from alcohol and pair fed controls ranged between 27 and 38% using GDH as a marker enzyme.

Mitochondria Respiration

The rate of oxidation of succinate (3.3 mM), glutamate-malate (0.33mM) and acetaldehyde (100μM) was measured polarographically at 30°C with a Clark-type oxygen electrode in a medium (3.0 ml) containing 0.25 M sucrose/20 mM triethanolamine (pH. 7.4) 10 mM potassium phosphate (pH 7.4) 5.0 mM $MgCl_2$/20 mM KCl/3 mg mitochondrial protein (10).

Calcium Metabolism in Mitochondria

a) The transport of Ca^{2+}

Calcium transport was measured using the "quench" techniques described by Reed and Bygrave (11,12). In each type of transport study, calcium uptake was terminated ("quenched") by addition of a quench solution (0.5mM EGTA, 3μM ruthenium red, 150mM KCl, pH 7.4) to an equal volume of incubation mixture.

b) Initial Rate of Uptake of Ca^{2+}

Initial rates of calcium transport were assessed at 4°C using varying amounts of total calcium (2000, 1500, 1000, 500, 100 and 50 μM). 0.1 ml of mitochondria and 0.1 ml ^{45}Ca (2.5 μCi/μmol Ca^{2+}) were added to 1.8 ml of media containing 10mM NTA-Tris (pH 7.4), 0.25M sucrose and 2mM succinate. Final mitochondrial protein concentration was 0.8-1.2 mg/ml. Ten seconds after the addition of ^{45}Ca, 0.1 ml of the incubation mixture was added to 0.1 ml of the quench solution. When studied, acetaldehyde (100μM) or ethanol (10mM) was added to the incubation mixture 2 minutes prior to ^{45}Ca. The quenched mixture was filtered (Millipore, 0.45 μm pore size) and the retained radioactivity measured in a Beckman LS-250 liquid scintillation spectrometer. Plots of initial rate against free calcium concentration were sigmoidal (13) and evaluated using the Hill equation. S_{50} was determined where $\log v/_{Vmax-v}=0$.

c) Retention of Ca^{2+} by Mitochondria

Calcium retention was measured in the presence of 4mM K_2HPO_4 (14) and at a total (final) calcium concentration of 100μM. The reaction mixture (25°C) contained in a total volume of 5 ml: 230 mM sucrose 10mM KCl 5.2 mM HEPES pH 7.4 5mM succinate 4mM K_2HPO_4 approximately 1 mg/ml mitochondrial protein and, when used, either acetaldehyde (100μM) or ethanol (10mM). Mitochondria were preincubated in the transport media 2 minutes prior to addition of ^{45}Ca. Additions of acetaldehyde or ethanol were also made 2 minutes prior to ^{45}Ca. Aliquots of the incubation mixture were removed at 15 sec., 30 sec., 1,2,4 and 6 minutes, added to an equal volume of ice-cold quench solution, centrifuged for 2 minutes in an Eppendorf microcentrifuge and 0.1 ml of the supernatant counted for radioactivity. Mitochondrial ^{45}Ca was calculated by subtracting the dpm in the supernatant from the dpm in an equal volume (0.1 ml) of incubation mixture not subjected to centrifugation.

d) Efflux of Ca^{2+} from Mitochondria

EGTA-induced calcium efflux was determined by prewashing mitochondria for 3 minutes with ^{45}Ca; aliquots of the incubation mixture were removed just before and 1,2,4 and 6 minutes after addition of EGTA (final concentration 2 mM).

e) Calcium content of mitochondria

When total mitochondrial calcium was to be measured in an aliquot of liver, lanthanum (1mM) was added to the homogenizing media (15). The calcium content of deproteinized mitochondrial suspensions was measured by atomic absorption spectrophotometry (Perkin Elmer Model 5000).

Acetaldehyde metabolism by mitochondria

Mitochondria (1 mg/ml) were incubated with 100 μM acetaldehyde in the buffer (5 ml) used in studies of calcium transport. Following the addition of acetaldehyde, the flasks were closed and incubated with shaking at 37°C. Aliquots (0.2 ml) were removed from the incubation flask at 2,5 and 10 minutes. After

addition of 0.2 ml PCA/thiourea and dilution with 0.8 ml H_2O, the acetaldehyde concentration in these aliquots (together with appropriate acetaldehyde standards) was measured by peak heights in a Perkin-Elmer F-40 headspace gas chromatograph (Perkin-Elmer Corp., Norwalk, Conn.) (16).

Other Assays:

Protein concentration of mitochondria was determined by the method of Lowry et al. (17). Glutamate dehydrogenase in the homogenate and mitochondrial fraction was assayed spectrophotometrically at 340 nm (18).

RESULTS

In vitro effects of ethanol and acetaldehyde on calcium transport by mitochondria from chow-fed rats.

The amount of Ca^{2+} detected in the mitochondria isolated from control rats was 20.0±8.9 nmoles/mg of protein, and neither the addition of 10mM ethanol to the incubation media, nor the pretreatment of the animal with cyanamide altered significantly this value (Table 1). In contrast, the addition of acetaldehyde (100μM) significantly increased the retention of calcium (40±7.1 vs 20.0±8.9 $p<0.01$). This effect was completely abolished by pretreatment of the rats with cyanamide. To further demonstrate that this effect was related to acetaldehyde specifically and its metabolism, the retention of Ca^{2+} was measured when other substrates entering site 1 of the respiratory chain were oxidized (Figure 1). The action of acetaldehyde on Ca^{2+} retention was significantly greater than the effect of glutamate + malate when these substrates were used at concentrations having comparable effects on mitochondrial O_2 consumption. Acetaldehyde (but not ethanol) also increased the affinity for calcium in initial rate studies. Hill plots demonstrated a decrease in S_{50} from 7.3 to 6.1 μM. Pretreatment of rats

TABLE I

In Vitro Effects of Ethanol and Acetaldehyde on Calcium Retention by Hepatic Mitochondria from Control and Cyanamide Treated Chow-Fed Rats

	Ca^{2+} Retention+ nmol/mg of protein	
	Control	Cyanamide°
Additions	(5)	(5)
None	20.0±8.9	19.2±4.0
Ethanol (10mM)	21.5±4.3	18.5±5.1
Acetaldehyde (100μM)	40.0±7.1*	23.7±5.

+ Values are means ± SEM at 2 minutes after the addition of $^{45}Ca^{2+}$. Comparisons were made to control values by group t test.
° Cyanamide (5 mg/kg i.p.) was administered 90 minutes prior to sacrifice.
*$p<0.01$

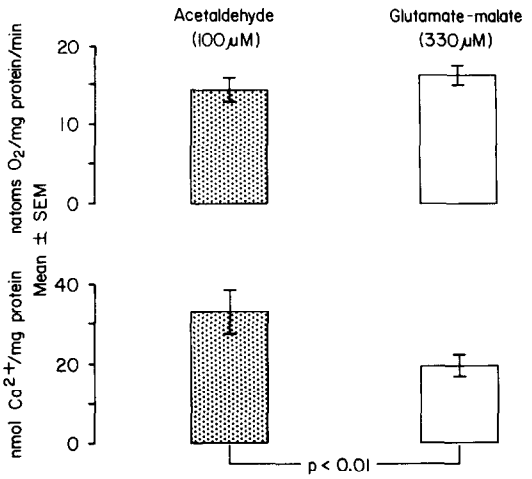
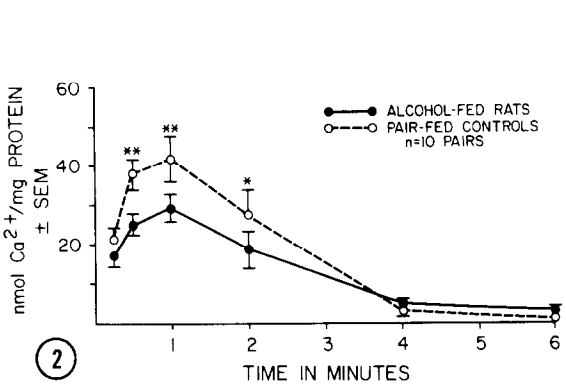


Figure 1: EFFECT OF ACETALDEHYDE AND NAD-DEPENDENT SUBSTRATES ON CALCIUM RETENTION AND MITOCHONDRIAL RESPIRATION
Calcium retention was assessed 2 minutes following the addition of either acetaldehyde (100μM) or glutamate-malate (.33mM). Mitochondrial oxygen consumption was measured in a medium (3 ml) at 30°C containing 0.25 M sucrose, 20mM triethanolamine, (pH 7.4), 10mM potassium phosphate (pH 7.4), 5.0mM $MgCl_2$, 20mM KCL and 3 mg mitochondrial protein.

with cyanamide (an inhibitor of mitochondrial acetaldehyde dehydrogenase) blocked the effects of low levels of acetaldehyde (100μM) on calcium retention (Table 1). Concomitantly, cyanamide decreased mitochondrial oxidation of acetaldehye by 80% (4.84±0.3 to 0.94±0.1 nmol/mg protein/min).

Calcium Transport by Isolated Mitochondria: Effect of Chronic Ethanol Consumption

Mitochondria isolated from chow-fed animal and liquid fed controls exhibited the same rates of Ca^{2+} transporting, indicating that the liquid diet per se did not affect this metabolic process. The Ca^{2+} retention by the mitochondria isolated from the ethanol-fed rats is presented in figure 2. In the preparation from both experimental groups, the initial rate (in first 15 sec) was essentially the same, but thereafter a significant difference was noted. The rate of Ca^{2+} uptake by the mitochondrial preparation from the ethanol-fed rats was significantly lower ($p<0.01$ at 1 min). The same mitochondria retained less Ca^{2+}, and as illustrated in figure 3, the decreased retention was associated with a significant increase in the efflux of calcium when EGTA was added to the incubation media. The total calcium content of mitochondria isolated from the control rats tended to be higher (5.44±0.15 vs 5.07±0.03 nmol Ca^{2+}/mg of protein)



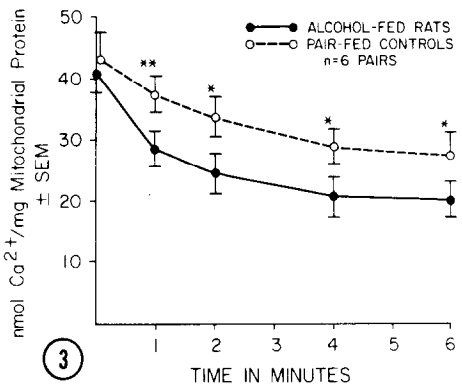


Figure 2: CALCIUM RETENTION BY MITOCHONDRIA FROM ETHANOL-FED (●) AND CONTROL (O) RATS. ^{45}Ca (100μM, 2.5μCi/μmol) was added to incubation mixture with aliquots quenched at 15 sec., 30 sec., 1, 2, 4 and 6 minutes.

Figure 3: EGTA-INDUCED EFFLUX OF CALCIUM FROM MITOCHONDRIA OF ETHANOL-FED (●) AND CONTROL (O) RATS. Mitochondria were preincubated with ^{45}Ca for 3 minutes. Aliquots were removed before (0 time) and 1, 2, 4 and 6 minutes after addition of EGTA (2mM). The results represent the mean ±SEM of duplicate samples from 6 pairs of animals. *$p<0.05$, **= $p<0.01$.

than in mitochondria from ethanol-fed rats but this difference was not statistically significant.

The effect of acetaldehyde on calcium retention by mitochondria from pair-fed control rats was comparable to that in chow rats. This effect of acetaldehyde was blunted in rats fed ethanol chronically. In contrast to a 66% increase in control rats ($p<0.01$), acetaldehyde increased calcium retention by only 37% in mitochondria from rats fed ethanol, a non-significant increase.

DISCUSSION

The data obtained from these in vitro studies suggest that the Ca^{2+} balance within the hepatocyte may be disturbed in rats maintained on a diet containing ethanol. In part, defective transport of calcium can be attributed to the effects of chronic ethanol intake on mitochondrial energy metabolism (19,20). However, steady state cycling of calcium reflects the concurrent operation of an independent efflux pathway (21,22) and the results depicted in Figure 3 suggest that impaired calcium uptake after ethanol feeding may also reflect augmented calcium efflux.

The in vitro effects of acetaldehyde on calcium transport were also assessed since impairment of several mitochondrial functions by chronic ethanol feeding has previously been linked to the toxicity of high levels of acetaldehyde (23). In

the present study, the effects of acetaldehyde were assessed at lower concentrations (100 μM) that are found in tissue during in vivo ethanol oxidation. Higher concentrations (1-30 mM) decreased both substrate and ATP-supported calcium uptake (23). However, it is unlikely that in vivo levels of acetaldehyde ever attain this magnitude.

In part, the effects of low concentrations of acetaldehyde appear to depend on mitochondrial metabolism of acetaldehyde. Cyanamide, a specific inhibitor of the low K_m mitochondrial aldehyde dehydrogenase blocked the stimulatory effect of acetaldehyde on calcium uptake and, concomitantly, reduced the rate at which acetaldehyde was metabolized by mitochondria. Although acetaldehyde may promote calcium uptake by serving as a respiratory substrate, additional factors are probably involved since its effects exceeded those of other NAD-dependent substrates. In this context, acetaldehyde increased the affinity for calcium in initial rate studies indicating a possible alteration of the calcium carrier. Finally, the blunted effect of acetaldehyde on mitochondria from ethanol-fed rats probably relates to their decreased capacity to oxidize acetaldehyde (16).

Since mitochondria play a predominant role in regulating the distribution of intracellular calcium, perturbations resulting from ethanol or acetaldehyde may upset steady-state levels of this cation. However, the relationship between changes in mitochondrial calcium transport and alcohol induced liver injury remains to be established.

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