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Effect of Ethionine on Hepatic Mitochondrial and Microsomal Calcium Uptake

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Ethionine, an ethyl analog of methionine, produces a variety of physiological and pathological effects in animals. These range from acute effects in the liver, kidney, pancreas, and other organs to liver carcinogenesis. Female rats when injected with ethionine exhibit a rapid decrease in hepatic adenosine triphosphate levels (Villa-Trevino et al, 1966) followed by a marked inhibition of RNA and protein synthesis and accumulation of triglycerides (villa-trevino et al, 1963, Farber et al, 1964). Ethionine has been used as a specific model to study the effects of toxic agents under the conditions of impaired mitochondrial function. DL-ethionine administration to rats produces partial or total uncoupling of mitochondrial respiration in the absence of morphologic evidence of mitochondrial injury (Wilson et al, 1986).

Since calcium transport in mitochondria and microsomes is ATP dependent, it becomes interesting to find out if ethionine administration has any effect on subcellular calcium transport. Calcium has recently gained an increased controversy regarding its role in chemical induced lethal cell damage. Certain groups believe that influx of extracellular calcium across the damaged plasma membrane might actually mediate the irreversible damage to the cell (Schanne et al, 1979, Farber, 1981) whereas according to others, entry of calcium into the cell is secondary to the damage (Smith et al, 1981; Farris and Reed, 1985). Present study was carried out to investigate the calcium transport in mitochondria and microsomes following ethionine administration. The effect of carbon tetrachloride on calcium uptake in ethionine treated rats was also studied.

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Materials and Methods

Female sprague-Dawley rats weighing 90-100g (Charles River Breeding Laboratories, Wilmington, MA) were housed in central animal facilities under a 12 hr photoperiod. The animals received commercial diet (Ralston Purina Chow Co., St Louis, MO) and regular water ad libitum. Ethionine at a dose of 1 mg/g body weight prepared in normal saline was given intraperitoneally. Control group received an equal volume of normal saline. The animals were killed at 1,3,5 and 7 hr after ethionine adminstration. Groups of animals also received ethionine followed by CCl_4 (0.2ml/kg) by the same route two hr later. The animals were killed one hr after CCl_4 administration. The liver was removed, washed, weighed and homogenized in ice cold 0.25 M sucrose. The homogenate was centrifuged at 500 g for 5 minutes and the supernatant was respun for 20 min at 6000 g. The resulting pellet was washed and suspended in 0.25 M surcose. The supernatant obtained from mitochondrial fraction was centrifuged at 105,000 g for 20 min. The pellet was rinsed and suspended in 0.25 M sucrose.

Calcium uptake was measured immediately after preparation of the samples. The assay system contained in a total volume of 1.5 ml, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ammonium oxalate, 5 mM sodium azide (for microsomes), 5 mM ATP, 40 uM $CaCl_2$ (0.1 uCi of $^{45}CaCl_2$/ml, New England Nuclear Corp.) and 30 mM histidine-imidazole buffer, pH 7.4 (Moore et al, 1976). Reaction was started by addition of the tissue fraction. Final protein concentrations were 80-100 ug/ml for microsomes and 40-60 ug/ml for mitochondrial incubations. The incubation was carried out at 37° C with reciprical shaking. Aliquots (400 ul) were removed at 1, 2 and 3 min. of incubation for mitochondria and 10, 20 and 30 min for microsomes, filtered through 0.45 u filters (type HA, Millipore Corp.) and washed with 3 ml of ice cold 0.25 M sucrose. ^{45}Ca in the filters was then measured using liquid scintillation counter in 5 ml aquasol (New England Nuclear Corp.). Calcium uptake was expressed as nmoles ^{45}Ca/mg protein/min. The uptake was calculated from 1 min to 3 min incubation period in case of the mitochondria and 10 to 30 min in case of the microsomes. Initial ^{45}Ca uptake value was omitted for the purpose of calculation because of a large variation in different groups which probably was due to different non-specific bindings taking place during the early incubation period. Protein was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

In vitro effect of ethionine on mitochondrial and

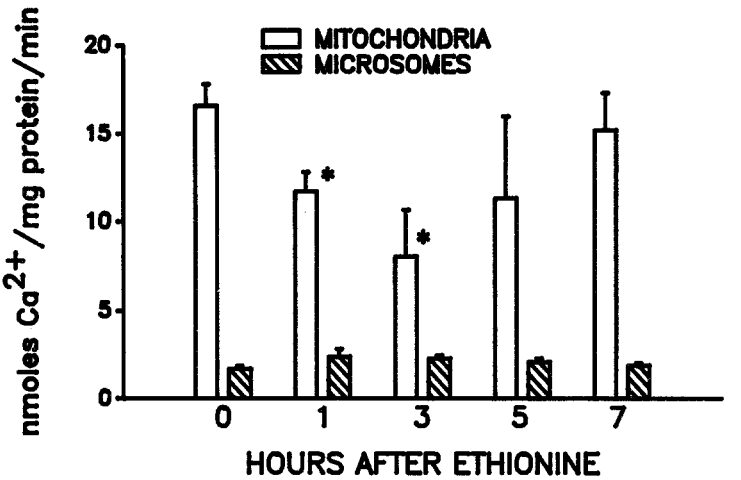

Figure 1. Female Sprague-Dawley rats (90-100g) were maintained on normal diet and water. The animals were starved overnight and received ethionine 1 mg/g in normal saline. Controls received an equal volume of normal saline. The animals were sacrificed at 1,3,5 and 7 hr after ethionine administration. Liver was removed, homogenized and mitochondria and microsomes were isolated by differential centrifugation. ^{45}Ca uptake was measured in mitochondrial and microsomal suspensions prepared in 0.25 M sucrose. The results, expressed as nmoles Ca^{2+}/mg protein/min are mean $\pm$ S.D. of four to five samples in each group. Asterisks denote the significance of difference at $p < 0.05$ from zero hr normal saline controls.

microsomal calcium uptake was also studied. Ethionine at a concentration of 2.5 mM to 40 mM was added to the incubation mixture and the subcellular calcium uptake was measured as described earlier.

Analysis of variance and students t test were used to evaluate the significance of difference between groups at a significance level of $p < 0.05$. The treated groups were compared to normal saline controls or the corn oil controls.

RESULTS AND DISCUSSION

Calcium plays an important role in regulating many cell functions and therefore, the levels of intracellular

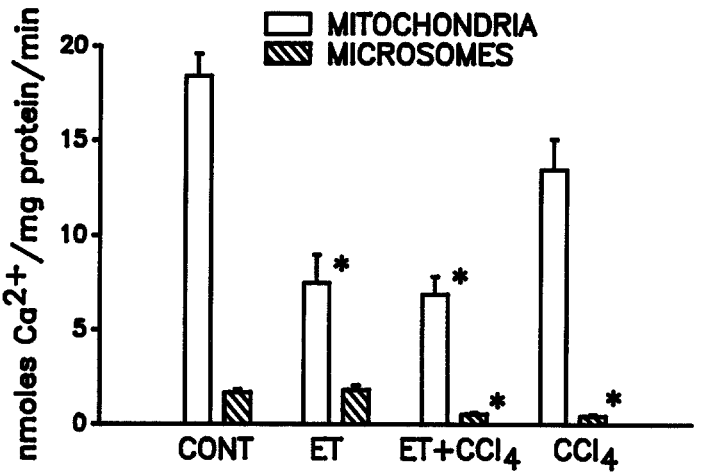

Figure 2. Mitochondrial and microsomal calcium uptake. The animals after overnight starvation received ethionine 1mg/g in normal saline. They received CCl_4 (0.2ml/kg) prepared in corn oil (1ml/kg) two hr later. The animals were killed one hr after CCl_4 administration. Appropriate controls received corn oil alone, normal saline alone or CCl_4 alone. Mitochondria and microsomes were isolated as described under "methods" and ^{45}Ca uptake was measured. Asterisks denote the signifcance of difference at $p < 0.05$ from controls.

calcium must be controlled and regulated. Higher levels of free calcium are known to affect many important physiologic processes. Mitochondria and microsomes play an important role in regulating the cell calcium (Bygrave, 1978; Becker et al, 1980). Calcium uptake in these organelles is ATP dependent. The extracellular concentration of calcium is 1000 fold higher than the intracellular. Any damage to plasma membrane will therefore, result in an influx of calcium. Mitochondria and microsomes sequester any excess calcium to maintain normal cytosolic levels.

Ethionine decreases hepatic ATP levels by 80-90% (Villa-Trevino et al, 1966; Higgins et al, 1978) and causes many specific biochemical and morphological changes (Farber, 1963; Farber et al, 1973). There is also a strong inhibition of RNA and protein sysnthesis (Villa-Trevino et al, 1966). It does become apparent that under such severe conditions many cellular functions including calcium uptake in mitochondria and microsomes might be greatly affected. Mitochondrial

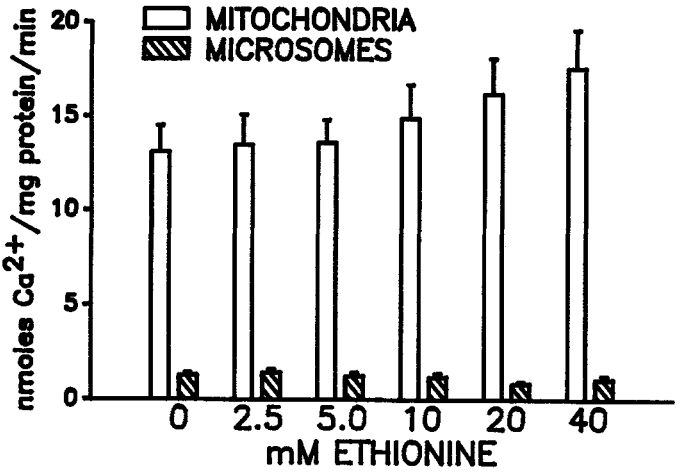

Figure 3. Effect of in vitro ethionine on mitochondrial and microsomal ^{45}Ca uptake. Ethionine at a final concentration of 2.5, 5.0, 10, 20 and 40 mM was added to the incubation medium described in "Methods" and mitochondrial or microsomal ^{45}Ca uptake was measured. No significant effect was observed.

calcium uptake decreases significantly at 1 and 3 hr after ethionine administration and recovers to near normal by seven hr (Fig. 1). According to Villa-Trevino et al (1963, 1966) ATP levels remain suppressed even after 5 or 7 hr following ethionine. It would appear that liver can withstand low levels of ATP and other disturbances for many hours without the loss of viability (Robinson and Seakins, 1962; Harris et al, 1968; Verbin et al, 1968). Mitochondria probably reach a stage of steady state in compromized conditions. Mitochondrial calcium uptake is significantly inhibited in the animals treated with ethionine and CCl_4 combination (Fig. 2). Ethionine by itself inhibits mitochondrial calcium uptake by 50%. It is possible that this inhibition is related to the effect on ATP. CCl_4 does not show any significant effect (Fig. 2) at the time point studied.

Microsomal calcium uptake was not affected significantly by ethionine (fig. 1). Ethionine administration produces endoplasmic reticulum membrane alterations and also affects cytochrome P-450 levels (Kisilevsky and Weiler, 1974, 1976; Chen and Smuckler,

1978). Previous studies indicate that the agents which produce free radicals and thereby destroy endoplasmic reticulum also significantly inhibit microsomal calcium uptake (Moore et al, 1976; Lowrey et al, 1981; Agarwal and Mehendale, 1986). Ethionine administration did not affect microsomal calcium uptake at any time point (Fig. 1). Administration of CCl_4 to ethionine pretreated rats significantly inhibited microsomal calcium uptake (Fig. 2). This inhibition does not seem to be due to ethionine pretreatment but due to CCl_4 itself. Bioactivation of CCl_4 produces $\cdot CCl_3$ free radical and lipid peroxidation which is probably responsible for the inhibition of microsomal calcium uptake (Moore et al, 1976).

Addition of ethionine in vitro did not significantly affect mitochondrial or microsomal calcium uptake at any concentration (Fig. 3). Microsomal calcium uptake showed a slight inhibition by 20mM and 40mM ethionine but it was not significant.

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