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Effect of Alpha-naphthyl Isothiocyanate and CCl_4 Interaction on Hepatocellular Calcium Transport

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Alpha-naphthyl isothiocyanate (ANIT), and CCl_4 are both hepatotoxic agents but with different mechanisms of action. The former produces its effect primarily by intrahepatic cholestasis (Goldfarb et al, 1962) while the latter is known to produce liver necrosis. In recent years, much attention has been focused on the disruption of intracellular calcium homeostasis as a common ultimate mechanism of the chemical induced irreversible hepatocellular damage (Schanne et al, 1979, Farber, 1981). Calcium is known to accumulate in necrotic tissues. The extracellular fluid is rich in calcium (10^{-4}M) whereas the concentration of the intracellular calcium is much lower (10^{-6} - 10^{-7}M) (Rasmussen, 1970). Therefore, it is believed that any damage to plasma membrane will result in an influx of calcium. Calcium ions are biologically very active, being capable of considerable disruption of metabolic order.

Agents such as CCl_4, $BrCCl_3$ which cause hepatocellular necrosis have also been reported to perturb the intracellular calcium homeostasis (Moore et al, 1976, Farber, 1981, Agarwal and Mehendale, 1984, 1984a, 1986, Prakash and Agarwal, 1988). Chemicals like ethanol, isopropanol, phenobarbital, chlordecone etc. potentiate this effect of CCl_4 (Agarwal and Mehendale, 1984, 1984a, 1986, Lowrey et al 1981, Moore and Ray 1983). The present study was designed to determine the effect of intrahepatic cholestasis as produced by a single toxic dose of ANIT on CCl_4 induced hepatotoxicity and hepatocellular calcium uptake. ^{45}Ca uptake in the isolated mitochondria and microsomes using three

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different doses of CCl_4 known to be toxic was measured in ANIT pretreated animals. Calcium ATPase in these organelles was assayed. Microsomal cytochrome P-450 and the levels of serum GPT and Bilirubin were also determined.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200-225 g (Charles River Breeding Laboratories, Wilmington, MA) were housed in central animal facilities and maintained on commercial food and regular water ad libitum. Groups of animals received 100 mg ANIT/kg ip in corn oil vehicle (1 ml/kg). CCl_4 in the doses of 0.5, 1.0 and 1.5 ml/kg in corn oil vehicle was administered ip 24 hr following ANIT. Controls received corn oil alone. The animals were sacrificed 24 hr after CCl_4 administration. The animals treated with ANIT were sacrificed 24 or 48 hr after the dose. The livers were removed washed and homogenized in a medium containing 20 mM sucrose, 220 mM mannitol and 2 mM HEPES. Mitochondria and microsomes were isolated by differential centrifugation. The pellets were washed and suspended in the homogenizing medium.

The in vitro ^{45}Ca uptake in the mitochondria and microsomes was measured immediately after preparation of the samples according to Moore et al (1976). Final protein concentrations in the incubation medium were 80 - 100ug/ml for microsomes and 40 - 60 ug/ml for mitochondrial assays. Aliquots were removed at 5, 10 and 15 min of incubation for mitochondria and 10, 20 and 30 min for microsomes and filtered through 0.45u filters Type HA, Millipore Corp.). Preliminary studies indicated that in the case of mitochondria, no significant changes in the ^{45}Ca uptake take place after CCl_4 at 1 to 3 min incubation period (Prakash and Agarwal, 1988). Mitochondria have been reported to sequester calcium in a biphasic manner - an initial rapid uptake phase which lasts 3 min followed by a slower phase of uptake (Kauffman and Lardy, 1980) indicating two different pools of calcium in the mitochondria. The slower uptake phase represents most of the exchangeable calcium and therefore, it is more appropriate to conduct ^{45}Ca uptake measurements in the mitochondria at 5 to 15 min incubation period (Prakash and Agarwal, 1988). Microsomes on the other hand, sequester calcium more slowly over a longer period of time and hence the incubation was carried out over a 30 min period (Moore et al, 1976). ^{45}Ca in the filters was measured in liquid scintillation counter in 10 ml aquasol^R (New England Nuclear Corp.). Protein was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

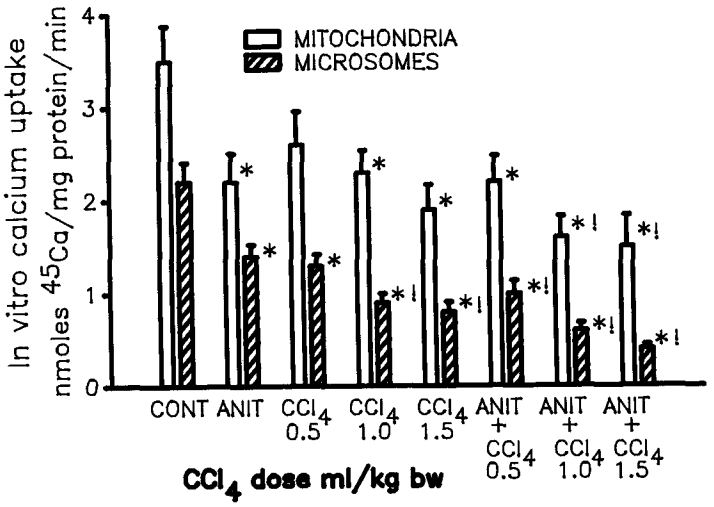

Fig. 1. In vitro ^{45}Ca uptake in isolated mitochondria and microsomes. Male Sprague-Dawley rats were administered ANIT, CCl_4 or a combination of ANIT and CCl_4. The liver mitochondria and microsomes were isolated by differential centrifugation and ^{45}Ca uptake was measured in the organelles as described. Results are expressed as mean + SEM of 4 to 5 samples from different animals. `*' denote the significance of difference at $p < 0.05$ from corn oil controls whereas `!' denote the significant difference at $p < 0.05$ from ANIT alone treated animals.

Ca^{++}-ATPase activity in the mitochondrial and microsomal fractions was determined by measuring the inorganic phosphate (Pi) liberated from ATP hydrolysis as described by Trottman et al (1985). The Pi liberated was estimated by the method of Lowry and Lopez (1964). Cytochrome P - 450 in the microsomal fraction was measured by the method of Omura and Sato (1964). Serum GPT and bilirubin were determined using Sigma diagnostic kits (Sigma Chemical Co., St. Louis, MO).

RESULTS AND DISCUSSION

Calcium has been known for many years to accumulate in the livers of animals poisoned by CCl_4 exhibiting a marked alteration in the intracellular calcium homeostasis (Moore et al, 1976, Farber, 1981, Agarwal and Mehendale, 1984, 1984a, 1986). A significant inhibition of mitochondrial and microsomal ^{45}Ca uptake was observed at 24 or 48 hr after ANIT. The inhibition was the same at both 24 and 48 hr and hence the data for only 48 hr ANIT has been presented (Fig. 1). CCl_4 by itself produced a significant decrease in the microsomal calcium uptake at all the three doses.

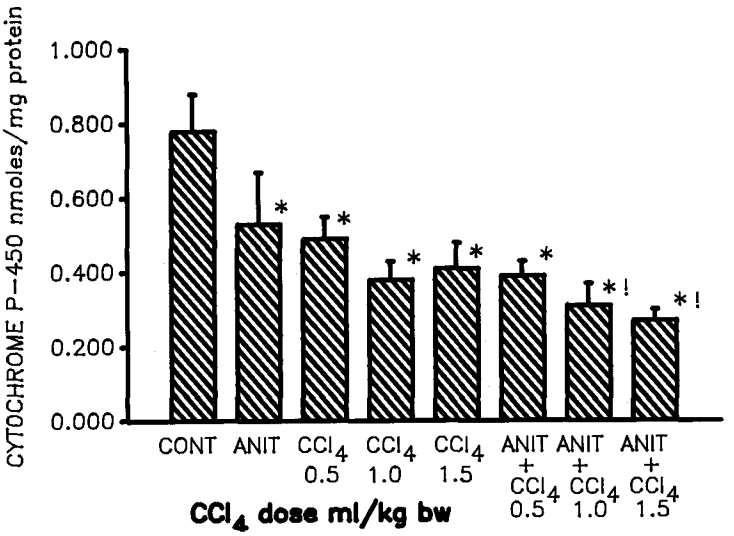

Fig. 2. Cytochrome P - 450 in the microsomal fraction. The microsomes were isolated at 105,000g for 20 min and the pellet suspended in tris-KCl buffer. Results are mean + SEM of 4 - 5 animals. `*' denote the significance of difference at $p < 0.05$ from corn oil controls and `!' denote the significant difference at $p < 0.05$ from ANIT treated animals.

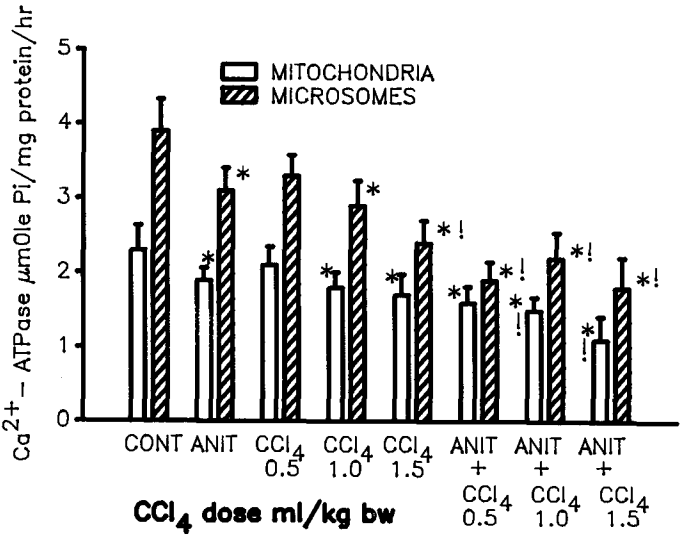

Fig. 3. Ca^{2+} - ATPase in the mitochondria and microsomes. The Ca^{2+} - ATPase was determined by the difference of Ca^{2+}, Mg^{2+} - ATPase and Mg^{2+} - ATPase by the addition of EGTA in the incubation medium. Results are mean + SEM of 4 - 5 animals. `*' denote the significance of difference at $p < 0.05$ from corn oil controls and `!' denote the significant difference at $p < 0.05$ from ANIT treated animals.

Mitochondrial calcium uptake was decreased only by 1.0 and 1.5 ml CCl_4/kg as reported earlier (Moore et al, 1976). The animals treated with ANIT and CCl_4 combination at doses of 1.0 and 1.5 ml/kg exhibited a further decrease in the calcium uptake when compared to CCl_4 alone or ANIT alone. No significant decrease was observed at 0.5 ml CCl_4/kg dose when compared to the animals treated with ANIT alone. The results indicate that although there is an additive effect of ANIT and CCl_4, the intrahepatic cholestasis as measured by increase in serum direct bilirubin does not seem to potentiate the effects of CCl_4 on the subcellular calcium uptake.

ANIT significantly increased the total bilirubin as well as the direct reacting (conjugated) bilirubin in the

Table 1. Effect of ANIT and CCl_4 interaction on serum GPT and bilirubin levels.

Treatment	GPT	Bilirubin	
	SF Units/ml	Total	Direct
Control	35 ± 5	0	0
ANIT 100mg/kg[a]	138 ± 16*	2.8 ± 0.4*	1.8 ± 0.2*
CCl_4 0.5ml/kg[b]	156 ± 20*	0.5 ± 0.2*	0.1 ± 0.04*
CCl_4 1.0ml/kg[b]	270 ± 44*	1.2 ± 0.3*	0.3 ± 0.1*
CCl_4 1.5ml/kg[b]	456 ± 80*	1.4 ± 0.4*	0.5 ± 0.1*
ANIT + CCl_4 0.5 ml/kg[c]	186 ± 30	3.3 ± 0.7	2.2 ± 0.4
ANIT + CCl_4 1.0 ml/kg[c]	423 ± 55!	3.6 ± 0.6!	2.7 ± 0.4!
ANIT + CCl_4 1.5 ml/kg[c]	780 ± 124!	3.8 ± 0.7!	2.9 ± 0.5!

[a] Animals sacrificed 48 hr after ANIT
[b] Animals sacrificed 24 hr after CCl_4
[c] Animals given ANIT followed by CCl_4 24 hr later; sacrificed 24 hr after CCl_4
* Denotes the significance of difference at $p < 0.05$ from corn oil controls
! Denotes significance of difference at $p < 0.05$ from ANIT treated animals.

serum (Table 1). Increase in serum direct bilirubin following ANIT administration was observed earlier by Goldfarb et al (1962). Additive effects of ANIT and CCl_4 were evident in the bilirubin levels also. CCl_4 by itself as reported earlier (Wooley et al, 1979) did not produce high levels of direct reacting bilirubin.

Serum GPT levels were higher after CCl_4 than after ANIT. Significant increases over ANIT alone or CCl_4 alone were apparent in the groups of animals receiving the combination of ANIT and CCl_4 (Table 1).

Reduction of cytochrome P - 450 by ANIT (Fig. 2) suggests a decreased bio-activation of CCl_4 in ANIT pretreated animals which might be responsible for the lack of potentiation of hepatotoxicity in ANIT + CCl_4 groups. Most agents which potentiate CCl_4 induced hepatic damage also induce cytochrome P - 450 with a consequent elevation in the CCl_4 bioactivation. Despite decreased cyt. P - 450, ANIT + CCl_4 group exhibited higher toxic damage as determined by serum bilirubin and GPT (Table 1).

A significant decrease in the Ca^{++} - ATPase activity was observed following ANIT or CCl_4 administration (Fig. 3). Calcium is an essential cellular component required for many cellular functions and is maintained in the organelles in a dynamic state based on the equilibrium between its uptake and release (Rasmussen, 1970). Mitochondrial and microsomal calcium pumps serve to regulate the intracellular calcium homeostasis (Becker et al, 1980). The activity of Ca^{++} - ATPase and calcium uptake are the two interlinked processes involved in calcium maintenance. Inhibition of these systems suggest a functional impairment of the cell. Whether the changes observed in the calcium uptake and ATPase are due to the specific effects of ANIT or CCl_4 on the liver mitochondrial and microsomal function, mitochondrial respiration etc. needs to be further evaluated. It is also possible that the morphological and biochemical alterations in the mitochondria and microsomes might affect the calcium transport system.

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