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Studies on calcium transport during carbon tetrachloride mediated hepatotoxicity in mice

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Administration of carbon tetrachloride (CCl_4*) to experimental animals or isolated hepatocytes causes acute hepatocellular necrosis. Although the underlying mechanisms are not completely understood, arguments have been put forth for [1] and against [2] the theory that CCl_4 toxicity is mediated through altered hepatocellular calcium homeostasis. The calcium homeostasis theory is based upon observations that the necrosis which develops subsequent to CCl_4 exposure is preceded by impaired calcium sequestering activity in liver endoplasmic reticulum (ER) and plasma membrane (PM) vesicles and by elevated cytosolic calcium concentration in isolated hepatocytes [2–4]. These effects may initiate a series of events resulting in loss of cell viability [5].

Many studies of ER and PM calcium uptake activities subsequent to CCl_4 administration *in vivo* have utilized doses of CCl_4 for which solvent effects unrelated to metabolic activation and subsequent hepatotoxicity of CCl_4 have been demonstrated [6]. Although species variability has been demonstrated for CCl_4 toxicity, such species effects would be due to differences in metabolic activation, rather than the purely physical solvent effects. The current studies were performed to determine whether reductions in ER or PM calcium uptake occur and correlate with hepatotoxicity at low CCl_4 doses which are unlikely to produce effects through direct solvent action. The associated changes in protein thiol content and calcium-dependent ATPase activity in reduced calcium uptake activity were also examined.

Methods

Animals and hepatotoxicant administration. Male ICR Swiss mice weighing 30–35 g were obtained from Harlan Laboratories (Indianapolis, IN). Animal care procedures were in accordance with recommendations described in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Academy of Sciences (DHEW Publication No. (NIH) 78-23, revised 1978). Animals were provided Purina 5001 Standard Rodent Diet and tap water *ad lib.* until use. CCl_4 was administered by intraperitoneal injection in a corn oil vehicle. Prior to use, the corn oil was extracted with methanol to remove antioxidants such as tocopherols, and then rendered peroxide-free by passage through a ferrous Dowex AG 1-X8 column [7]. Control animals received vehicle only. Following CCl_4 or vehicle exposure, animals were killed by cervical dislocation either at 2 hr for studies of calcium uptake or at 24 hr for assessment of liver injury. Livers (dissected free of gall bladders) were removed and rinsed in 0.9% (w/v) NaCl. All subsequent operations were performed at 0–4°.

Preparation of subcellular fractions. Liver plasma membrane vesicles were prepared using a modification of the procedure of Epping and Bygrave [8] as previously described [9]. Liver microsomes were prepared as described by Moore *et al.* [10] and then were suspended in a buffer consisting of 0.125 M sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 5 mM HEPES-KOH (pH 6.8). Microsomal and plasma membrane vesicles were utilized for subsequent assays immediately after preparation.

Microsomal and plasma membrane calcium uptake. Microsomal calcium uptake activities were determined with a filtration technique using $^{45}CaCl_2$ as described by Moore *et al.* [10]. Plasma membrane calcium uptake activities were determined as previously reported [9].

Microsomal calcium-dependent ATPase. Microsomes (1 mg protein/ml) were incubated at 37° in 1 ml of a reaction mixture containing 100 mM KCl, 30 mM imidazole-histidine (pH 6.8), 10 μM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM sodium azide, 0.5 μM ruthenium red, and 1 mM ATP. Reactions were initiated with ATP and terminated after 15 min by addition of 0.1 ml of 50% trichloroacetic acid. The mixtures were then centrifuged, and the amount of inorganic phosphate in the supernatant solution was determined colorimetrically as described [9]. The calcium-dependent ATPase contribution was determined by subtracting the ATPase activity observed in parallel incubations in which $CaCl_2$ was omitted and replaced with 2 mM EGTA.

Other procedures. Microsomal protein thiol content was determined by the method of Albano *et al.* [11]. Serum alanine aminotransferase (ALT) activity was determined as described by Segal and Matsuzawa [12]. Necrosis was determined by histological examination of liver sections and quantitated by assigning a score of 0–5, 0 indicating normal histology. Protein was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL) according to the technical procedure supplied by the manufacturer. Statistical analyses were conducted using Student's *t*-test, with $P < 0.05$ established for significance.

Results and discussion

Dose-related liver injury, indicated by plasma ALT activity and histopathological evidence of liver necrosis, was detected 24 hr following exposure to CCl_4 doses of 20–200 μl/kg body weight (Table 1). Although 5 μl/kg body weight produced no significant ALT increase or histopathological necrosis, at 2 hr following exposure to ≥ 20 μl/kg body weight, the ER calcium sequestering activity was reduced by two-thirds. This contrasts with only a 20% decrease at the lower dose (5 μl/kg) and suggests that a substantial inhibition of the microsomal calcium pump may be required in order for this effect to contribute to the toxicity of CCl_4.

Tsokos-Kuhn *et al.* [4] detected a marked reduction in calcium sequestering activity in plasma membrane vesicles subsequent to a CCl_4 dose of 2 ml/kg body weight. As indicated in Table 2, we also noted an inhibition of plasma membrane calcium sequestration at this dose, and we speculate that this is a solvent effect, since a lower but hepatotoxic CCl_4 dose of 50 μl/kg body weight did not reduce plasma membrane calcium sequestering activity. Therefore, in contrast to microsomes, non-solvent mediated CCl_4 hepatotoxicity does not appear to be associated with inhibition of plasma membrane calcium sequestering activity 2 hr subsequent to the dose.

The inhibition of calcium transport activity in liver subcellular organelles which is produced by various chemicals is reportedly associated with reductions in protein thiol content and CaATPase activity in these organelles [13–15]. However, the data in Table 3 indicate that protein thiol content and CaATPase activity were not reduced in micro-

* Abbreviations: ER, endoplasmic reticulum; CCl_4, carbon tetrachloride; CaATPase, calcium activated ATPase; ALT, alanine aminotransferase; PM, plasma membrane; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and EGTA, ethyleneglycolbis(aminoethylether)-tetra-acetate.

Table 1. Parameters of tissue and microsomal response to CCl_4 administration

CCl_4 dose (μl/kg)	Plasma ALT (μmol/min·ml)	Liver necrosis (histological scale)	Microsomal calcium (uptake (% control)
0 (control)	0.04 ± 0.01	0	100
5	0.05 ± 0.01	0.3 ± 0.2	80.0 ± 6.7
20	2.29 ± 0.43*	3.0 ± 0.7*	33.0 ± 2.6*
200	7.50 ± 0.59*	4.5 ± 0.3*	34.8 ± 4.1*

Mice were treated with CCl_4 at the indicated doses as described in Methods. Animals were killed at 24 hr for measurement of plasma alanine aminotransferase (ALT) and evaluation of histology; animals were killed at 2 hr for measurement of microsomal calcium uptake. Values are means ± SEM for four to seventeen animals per group. The histological scale for centrozonal liver necrosis was: (0) none; (3) moderate; and (5) severe. For each animal, several sections were prepared, and about fifteen central vein regions were examined. The control value for microsomal calcium uptake was 67 ± 4 nmol/mg protein·15 min.

* Significantly different from control, $P < 0.05$.

Table 2. Parameters of tissue and plasma membrane response to CCl_4 administration

CCl_4 dose (μl/kg)	Plasma ALT (μmol/min·ml)	Plasma membrane calcium uptake (% control)
0 (control)	0.04 ± 0.01	100
50	6.08 ± 0.59*	109 ± 19
2000	9.41 ± 1.6*	69 ± 19*

Mice were administered CCl_4 at the indicated doses as described in Methods. Animals were killed at 24 hr for measurement of plasma ALT and at 2 hr for measurement of plasma membrane calcium uptake. Values are means ± SEM for three to six animals. The control value for plasma membrane calcium uptake was 1.78 ± 0.13 nmol/mg protein·3 min.

* Significantly different from control, $P < 0.05$.

Table 3. Parameters of hepatic microsomal response to CCl_4 administration

CCl_4 dose (μl/kg)	Microsomal calcium uptake (% control)	Microsomal Ca-ATPase (% control)	Microsomal protein-SH (% control)
0 (control)	100	100	100
200	34.8 ± 4.1*	85.1 ± 9.8	117 ± 11

Mice were administered CCl_4 at the doses shown, as described in Methods. The results are expressed as the mean ± SE of six animals in each group. Control values were 144 ± 5 nmol SH/mg protein, 67 ± 4 and 256 ± 14 nmol/mg protein·15 min for protein thiol content, calcium uptake, and CaATPase activities respectively.

* Significantly less than control, $P < 0.05$.

somes exhibiting a CCl_4-induced reduction in calcium uptake activity to 34.8% of control.

In summary, our data support the hypothesis that alterations in hepatocellular calcium homeostasis may contribute to the mechanism by which CCl_4 causes acute liver necrosis. The ability of liver ER to sequester calcium appears to be an early target of CCl_4-induced injury, whereas the plasma membrane is not similarly affected at early intervals. This does not rule out the possibility that alterations in the handling of calcium by the plasma membrane may contribute to subsequent stages of CCl_4-induced liver injury. The current data cannot be used to distinguish whether observed decreases in calcium sequestration by vesicle preparations represent changes in membrane permeability or specific inhibition of calcium transport activities. However, since alterations in calcium homeostasis induced by liver injury may involve modification of thiol residues of transport associated proteins, it is of interest that the current studies demonstrated marked inhibition of calcium sequestration by liver microsomes without reduction in either microsomal protein thiol content or CaATPase activity.

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Differential expression of glutathione transferases by native and cultured human lymphocytes

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The glutathione transferases (GSTs) are a unique group of dimeric proteins which catalyse the conjugation of reduced glutathione (GSH) with a variety of compounds possessing an electrophilic centre [1]. Many chemical carcinogens such as the polycyclic aromatic hydrocarbons are thought to exert their effect through the formation of highly reactive epoxide intermediates which can be detoxified by GST-catalysed formation of GSH conjugates [2] or may lead to the initiation of carcinogenesis by covalent interaction with nucleic acids or critical proteins. Interindividual differences in the expression of cytochromes P-450 and GSTs could therefore have important implications in the susceptibility of individuals to some forms of cancer.

The human GSTs are subject to a tissue-specific distribution, and on the basis of differing immunological and physical properties, substrate specificities and protein structure, these isoenzymes can be assigned to one of three classes: alpha, mu or pi [3]. Class alpha forms, for example, GSTs α–ε were originally described in human liver [4] as the predominant forms present whilst the presence of up to thirteen immunochemically identical basic hepatic forms has now been reported [5]. Two basic forms have been isolated from human skin, one of which (pI 8.5) was found to be immunologically related to GST α–ε and the other (pI 9.9) showed greater similarity to rat GST 2-2 than other human class alpha forms [6]. However, it appears that the predominant GSTs of extrahepatic tissues such as placenta [7], lung [8], uterus [9] or erythrocytes [10] are generally members of class pi. It has been reported that as much as 97% of GST activity in lung [8] and 85–90% activity of uterus [9] can be attributed to the presence of acidic forms.

Human GST-μ, a member of class mu with a "near-neutral" isoelectric point, has been isolated from adult liver [11] and more recently a protein having immunological identity with human hepatic GST-μ has been detected in peripheral blood mononuclear leukocytes [12]. Hepatic GST-μ shows a higher efficiency for conjugating epoxides such as benzo[*a*]pyrene 4,5-oxide (BPO) and styrene 7,8-oxide than either class alpha or pi forms and the expression of this form differs markedly between individuals [11]. The conjugation of *trans*-stilbene oxide with GSH in peripheral mononuclear leukocytes also displays a polymorphic distribution [13] where the GST form responsible is reported to be identical with hepatic GST-μ [12]. In addition, a recent study using a specific RIA method to measure class mu GST forms μ and γ in mononuclear leukocytes found that only 55% individuals expressed these forms [14]. There is now evidence to suggest that interindividual variations in the expression of class mu GSTs may be a factor in the susceptibility of cigarette-smokers to lung cancer [15].

We have been assessing the suitability of cultured lymphocytes such as Epstein–Barr virus (EBV) transformed B-cell lines and interleukin-2- (IL-2) dependent T-cells for use in investigations of GST activity with special regard to the polymorphism of class mu GSTs. We have previously reported marked differences in the rate of formation of GSH conjugates with BPO and 1-chloro-2,4-dinitrobenzene (CDNB) between freshly isolated and cultured lymphocytes [16, 17]. In this paper we have investigated the expression of GSTs by native and cultured human lymphocytes using Western immunoblotting.