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### 1,1-Dichloroethylene inhibition of liver endoplasmic reticulum calcium pump function

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1,1-Dichloroethylene (vinylidene chloride, 1,1-DCE) is a potent hepatotoxin that, in contrast to agents such as  $\text{CCl}_4$ , does not produce lipid peroxidation in liver membranes [1, 2]. Electron microscopic examination of livers from 1,1-DCE-treated, mature animals clearly demonstrates damage at the nucleus, plasma membrane and mitochondria [3, 4]. Again, in contrast to agents such as  $\text{CCl}_4$ , liver endoplasmic reticulum (ER) damage does not appear as an early morphological change after 1,1-DCE administration to mature rats [4]. Significant biochemical changes of ER function are thought to occur late in 1,1-DCE toxicity. Inhibition of the mixed function oxidase system (MFOS) and of glucose-6-phosphatase (G6Pase) in ER is not observed until 1,1-DCE-induced liver damage is well established [4]. However, a recent report shows that activity of an ER calcium transport pump is dramatically decreased 2 hr after 1,1-DCE administration [5]. The current study characterizes the time course of 1,1-DCE effect on a calcium transport system in liver ER and on ER calcium levels at times up to 4 hr after 1,1-DCE administration. The results demonstrate that dramatic changes of ER function occur very early after 1,1-DCE administration. This observation may suggest that, in common with  $\text{CCl}_4$  and other hepatotoxins, ER damage occurs early in the sequence of 1,1-DCE intoxication. This observation lends additional support to the hypothesis that alteration of intracellular calcium may initiate a series of events that produce cell death [5–8].

Male Sprague–Dawley rats (250–400 g) were used for this study. Animals were allowed free access to food and water throughout the experiments. 1,1-DCE (Aldrich Chemical Co., Milwaukee, WI) was diluted in corn oil and administered i.p. In some experiments, rats were pretreated with diethylmaleate (DEM, Sigma Chemical Co., St. Louis, MO) (0.6 ml/kg i.p.) 1 hr before 1,1-DCE. In other experiments, rats were pretreated with three doses of phenobarbital (J. T. Baker Chemical Co., Phillipsburg, NJ) (80 mg/kg) 72, 48 and 24 hr before 1,1-DCE. Calcium pump activity was determined in either a microsomal fraction [5, 6] or a 12,500 g supernatant fraction. All calcium uptake activity in the 12,500 g supernatant fraction could be attributed to the calcium pump in the microsomal fraction. Calcium pump activity was measured in the following medium: 100 mM KCl, 30 mM imidazole–histidine buffer

(pH 6.8), 5 mM  $\text{MgCl}_2$ , 5 mM ATP (pH adjusted to 6.8 with imidazole), 5 mM ammonium oxalate, 5 mM sodium azide, 20  $\mu\text{M}$   $\text{CaCl}_2$  ( $[^{45}\text{Ca}^{2+}]$ , 0.2  $\mu\text{Ci/ml}$ ) and 20–50  $\mu\text{g}$  microsomal protein (or equivalent 12,500 g supernatant fraction)/ml. The assay was initiated by addition of the membrane fraction to prewarmed assay medium (37°). At timed intervals, samples were removed, filtered through 0.45  $\mu\text{m}$  nitrocellulose cellulose filters, and  $[^{45}\text{Ca}^{2+}]$  was determined by liquid scintillation spectrophotometry. Liver and microsomal calcium was determined by atomic absorption spectrophotometry, as previously described [5]. Glucose-6-phosphatase activity was determined as described by Aronson and Touster [9]. Glutathione was determined as described by Jaeger *et al.* [10]. Lipids were extracted from the 12,500 g supernatant fraction after *in vivo* administration of 1,1-DCE, and lipid peroxidation was determined as conjugated dienes at 243 nm as described by Klaassen and Plaa [11]. Protein was determined by the Lowry method as described by Shatkin [12].

As demonstrated in Fig. 1, calcium pump activity was inhibited 45% within 20 min after 1,1-DCE administration. Pump activity continued to decline and was maximally (70%) inhibited by 4 hr. In a previous study, this nadir was reached by 2 hr [5]. 1,1-DCE administration depletes GSH [4, 10] and GSH depletion potentiates 1,1-DCE hepatotoxicity [10]. GSH is important in 1,1-DCE metabolism and probably serves as a detoxification route for a reactive 1,1-DCE metabolite [13–15]. Inhibition of the calcium pump paralleled loss of GSH (Fig. 1). However, GSH depletion in itself was not sufficient to produce calcium pump inhibition. Doses of DEM of up to 2.4 ml/kg did not result in calcium pump inhibition or release of microsomal calcium when animals were killed 1 hr after DEM administration. Other evidence of 1,1-DCE-induced, ER dysfunction is presented in Fig. 1. Calcium associated with the microsomal fraction began to decline within 20 min after 1,1-DCE administration and fell to 50% of control at 4 hr. This is similar to an effect produced by  $\text{CCl}_4$  or  $\text{CHCl}_3$  [5]. In contrast to these effects on ER calcium systems and in agreement with previous findings, 1,1-DCE did not alter microsomal G6Pase activity [5] or the level of conjugated dienes extracted from microsomes [2, 5] (Fig. 1).

Evidence of extensive liver damage was obvious at 4 hr. At this time, serum glutamic pyruvic transaminase activity

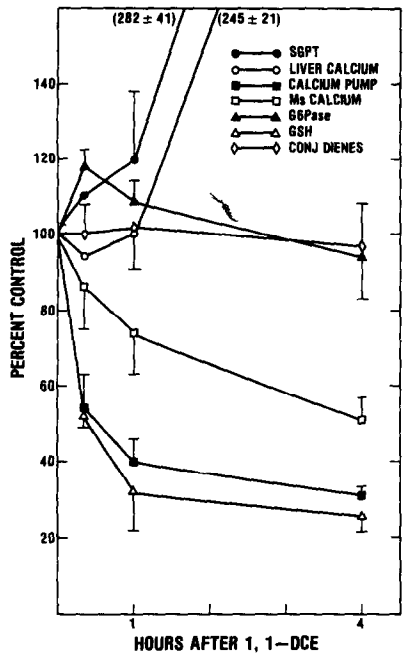


Fig. 1. Time course of effect of 1,1-DCE on rat liver. Rats were treated with 1,1-DCE (1.5 ml/kg, i.p.). All variables were determined as described in the text. Each point is the mean  $\pm$  S.E.M. for determinations performed on material from four to six rats.

(SGPT) had increased and calcium in the liver had increased (Fig. 1). Both changes represent breakdown of the hepatocyte plasma membrane with leakage of the cytoplasmic enzyme out of the liver and the beginning of a massive calcium influx. Calcium in the liver has been shown to increase 3-fold, 6–24 hr after 1,1-DCE [4, 5].

Additional evidence suggests a correlation between 1,1-DCE hepatotoxicity and calcium pump inhibition. Depletion of hepatic GSH with DEM potentiates 1,1-DCE hepatotoxicity [10] and calcium pump inhibition (Table 1). In this experiment hepatic GSH was reduced to  $22 \pm 3\%$  of control 1 hr after DEM (0.6 ml/kg) administration. The first experiment in Table 1 shows that liver microsomal calcium pump activity was inhibited to a significantly greater

extent in preparations from DEM-pretreated rats killed 1 hr after a moderately hepatotoxic dose of 1,1-DCE (0.3 ml/kg). The second experiment shows that control rats treated with 1,1-DCE at 0.03 ml/kg showed no evidence of hepatotoxicity 24 hr later adjudged by SGPT activity. However, the DEM-pretreated group exhibited marked hepatotoxicity (SGPT activity) and a marked increase of calcium pump inhibition. Phenobarbital induction of the MFOS has been reported to decrease [3] or to increase 1,1-DCE toxicity [16–18]. It has been shown that the effect of both phenobarbital induction and SKF-525A inhibition of the MFOS on 1,1-DCE toxicity differs with weight of the treated animals [18]. In the present study, phenobarbital induction clearly increased calcium pump inhibition. Liver microsomal calcium pump activity was inhibited  $35 \pm 4.9\%$  in control animals and  $70 \pm 5.3\%$  in phenobarbital-pretreated animals killed 1 hr after administration of 1,1-DCE (0.3 ml/kg). This difference was statistically significant ( $P < 0.01$ , Student's *t*-test). This would suggest that phenobarbital induction of the MFOS increased production of a 1,1-DCE metabolite or metabolites responsible for inhibition of the liver ER calcium pump and lethality.

Calcium has been suggested as a mediator of toxin action in the liver [5–8]. Certain studies suggest that inhibition of the liver ER calcium pump may be the initial insult that disrupts liver calcium homeostasis sufficiently to produce an injury that allows massive calcium influx [5–7]. With  $\text{CCl}_4$  and  $\text{BrCCl}_3$ , prompt inhibition of the ER calcium pump has been observed after *in vivo* administration [5, 6, 19]. This corresponds to the observation that the ER is the first site at which morphological evidence of  $\text{CCl}_4$  damage is observed [20]. However, disruption of ER morphology and of some ER functions does not occur early during the course of 1,1-DCE intoxication in mature rats [3, 4, 21]. The current study and previous work [5–7, 19] demonstrate that a calcium transport system in the ER is disrupted as an early event after  $\text{CCl}_4$  and 1,1-DCE. This disruption of calcium transport may ultimately lead to a breakdown of normal plasma membrane permeability barriers. This plasma membrane change would result in the massive accumulation of calcium observed after  $\text{CCl}_4$  [5, 6, 20] and 1,1-DCE [4, 5]. Although not addressed in this study, it is possible that the early morphological changes seen after  $\text{CCl}_4$  are a result of lipid peroxidation. In addition, there is some evidence to suggest that inhibition of both G6Pase and MFOS is dependent upon chlorinated hydrocarbon-induced lipid peroxidation [5, 6, 22, 23]. Because 1,1-DCE does not induce lipid peroxidation, it

Table 1. 1,1-DCE hepatotoxicity in diethylmaleate-pretreated rats\*

Group	Calcium pump activity (% inhibition)	SGPT activity (% control)
Experiment 1		
Control	$33 \pm 4$	ND†
DEM-pretreated	$57 \pm 4\ddagger$	ND
Experiment 2		
Control	$15 \pm 7$	$105 \pm 5$
DEM-pretreated	$41 \pm 2\ddagger$	$1130 \pm 236\ddagger$

\* In both experiments, diethylmaleate (DEM) was administered (0.6 ml/kg, i.p.) 1 hr before 1,1-DCE. In experiment 1, 1,1-DCE was administered (0.3 ml/kg) 1 hr before the animals were killed. Calcium pump activity was determined with the microsomal fraction and is expressed as the mean percent inhibition  $\pm$  S.E.M. for the determination in eight preparations. In experiment 2, 1,1-DCE was administered (0.03 ml/kg) 24 hr before the animals were killed. Calcium pump activity was determined with microsomal membranes and is expressed as mean percent inhibition  $\pm$  S.E.M. ( $N = 6-8$ ). SGPT activity was determined in serum samples and is expressed as the mean of percent control  $\pm$  S.E.M. ( $N = 6-8$ ).

† ND = not determined.  
‡  $P < 0.01$ , Student's *t*-test.

would be expected that 1,1-DCE could produce functional changes in both the ER calcium pump and ER contribution to calcium homeostasis without observable changes in ER morphology, MFOS activity or G6Pase activity.

In summary, this study shows that 1,1-DCE promptly inhibits a calcium homeostatic function of liver ER. The correlation with GSH depletion and the effect of MFOS induction on calcium pump inhibition suggest that this is a direct effect of a 1,1-DCE metabolite on the calcium pump. As a result of calcium pump inhibition, calcium released from the ER may serve to trigger changes that result in a massive influx of extracellular calcium and, ultimately, cytotoxicity.

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## Carbon disulfide hepatotoxicity and inhibition of liver microsome calcium pump

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Certain chlorinated hydrocarbon hepatotoxins inhibit the liver endoplasmic reticulum (ER,\* microsome) calcium pump after exposure *in vivo* or *in vitro* [1–3]. Some investigators suggest that disruption of calcium homeostasis and alteration of intracellular calcium distribution by these toxins may initiate a cascade of events that terminates in hepatic necrosis [1–5]. Because of a possible mechanistic role of calcium pump inhibition in hepatotoxin action, it is of interest to examine other classes of hepatotoxins for the ability to inhibit the liver ER calcium pump. CS<sub>2</sub> is a hepatotoxin that produces extensive centrilobular necrosis in phenobarbital-pretreated, starved rats, while in normal rats CS<sub>2</sub> produces fatty infiltration but little necrosis [6–8].

This compound is appropriate as a model of non-halogenated hepatotoxins because its toxic actions range from lipid accumulation in the liver to hepatocytic necrosis.

Male Sprague–Dawley rats (250–400 g) were used for this study. Animals were allowed free access to food and water throughout the experiments. CS<sub>2</sub> (Fisher Scientific Co., Fair Lawn, NJ) was diluted in corn oil and administered *i.p.* or orally. Rats were pretreated with phenobarbital (J. T. Baker Chemical Co., Phillipsburg, NJ) (80 mg/kg) 72, 48 and 24 hr before CS<sub>2</sub>. Microsomal calcium pump activity was measured in the following medium: 100 mM KCl, 30 mM imidazole–histidine buffer (pH 6.8), 5 mM MgCl<sub>2</sub>, 5 mM ATP (pH adjusted to 6.8 with imidazole), 5 mM ammonium oxalate, 5 mM sodium azide, 20  $\mu$ M CaCl<sub>2</sub> ([<sup>45</sup>Ca<sup>2+</sup>], 0.2  $\mu$ Ci/ml) and 20–50  $\mu$ g microsome protein (or equivalent 12,500 g supernatant fluid)/ml [1, 2]. The assay was initiated by addition of the membrane fraction to prewarmed assay medium (37°). At timed inter-

\* Abbreviations: ER, endoplasmic reticulum; G6Pase, glucose-6-phosphatase; SGPT, serum glutamic pyruvic transaminase; 1,1-DCE, 1,1-dichloroethylene; GSH, glutathione; and MFOS, mixed function oxidase system.