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1,1-Dichloroethylene Inhibition of Liver Microsomal Calcium Pump *in Vitro*

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Rat liver microsomes were preincubated with 1,1-dichloroethylene (1,1-DCE) and NADPH-generating system for 20 min, then ER calcium pump activity determine Calcium pump activity was inhibited as a function of 1,1-DCE concentration. 1,1-DC did not inhibit in the absence of a NADPH-generating system. Calcium pump activit was inhibited to a significantly greater extent by 1,1-DCE in microsomes isolated fro phenobarbital-pretreated rats than in microsomes from control rats. These studie suggest that the mixed function oxidase system generates a metabolite that attack the endoplasmic reticulum (ER) calcium pump. The time course of pump inhibition b 1,1-DCE and CCl_4 were compared *in vitro*. 1,1-DCE produced inhibition of the calciu pump at a substantially slower rate. CCl_4 at 1 μliter/ml inhibited calcium pump activit within 1 min. 1,1-DCE did not produce significant inhibition within 1 min, but produce $58 \pm 5\%$ inhibition by 20 min. CCl_4 but not 1,1-DCE, produced lipid peroxidation a judged by malonic dialdehyde production. It is possible that lipid peroxidation con tributes to the rapid course of inhibition produced by CCl_4. These studies suggest tha metabolic activation, but not lipid peroxidation, is a prerequisite for inhibition of th liver ER calcium pump by chlorinated hydrocarbon hepatotoxins *in vitro*.

1,1-Dichloroethylene (1,1-DCE,[2] vinylidene chloride) is a potent hepatotoxin encountered in our environment. In general, toxicity of 1,1-DCE may be considered quantitatively comparable to CCl_4 (1). However, qualitatively the actions of these compounds differ in certain aspects. Morphologically the first evidence of CCl_4-induced damage occurs at the liver endoplasmic reticulum (2). One of the first biochemical changes noted after CCl_4 administration is a marked increase of lipid peroxides in liver membranes (3). After 1,1-DCE there is little or no morphological evidence of early damage to the liver endoplasmic reticulum in matur rats (4) and there is no evidence that thi compound increases lipid peroxidation i *vivo* (5). Qualitatively other aspects of th action of these two chlorinated hydrocar bon hepatotoxins are similar. Previou work from our laboratory shows that bot CCl_4 and 1,1-DCE inhibit the liver endo plasmic reticulum (microsomal) calciu pump after *in vivo* administration. A prompt, dose-dependent inhibition of thi ATP-dependent calcium pump occurs af ter CCl_4 and 1,1-DCE (6, 7). Other labo ratories have demonstrated similar ef fects of other hepatotoxins (8, 9) and an oxia (10).

Inhibition of the endoplasmic reticulun calcium pump appears to be ne of th earliest effects of hepatotoxic chlorinate hydrocarbons in the liver (9). It is possibl that inhibition of this pump by hepato

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[2] Abbreviations used: 1,1-DCE, 1,1-dichloroethylene; TK buffer, 0.1 M KCl, 0.05 M Tris, pH 7.4; MFO, mixed function oxidase.

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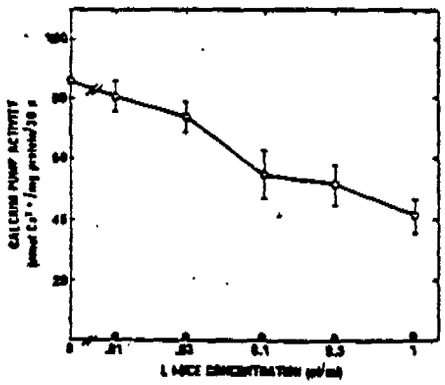

FIG. 1. Inhibition of liver microsome calcium pump *in vitro* by various concentrations of 1,1-DCE. After 20 min of incubation with the indicated concentration of 1,1-DCE and in the presence of (○) or absence (●) of a NADPH generating system, calcium pump activity was determined as described under Materials and Methods. Each point represents the mean ± SEM for the determination with microsome preparations isolated from three animals. No SEM is indicated for the experiments without a NADPH generating system because the SEM fell within the area occupied by the symbol.

oxins may disrupt calcium homeostasis n the liver and initiate a series of events hat lead to the massive calcium influx gins several hours after intoxica-

To examine the interaction between the ver endoplasmic reticulum calcium pump ith chlorinated hydrocarbon hepatotox- s more closely, we have characterized e *in vitro* effect of 1,1-DCE on the mi- osomal calcium pump. As part of this aracterization we have compared effects oduced by 1,1-DCE and CCl_4. Because 1-DCE does not increase lipid peroxides liver membranes this hepatotoxin al- us to demonstrate that inhibition of e liver endoplasmic reticulum calcium mp, *in vitro*, does not require peroxi- tion of membrane lipids.

MATERIALS AND METHODS

aterials. Male Sprague-Dawley rats (200–300 g, onic Farms) were used in this study. Unless oth- ise noted, rats were pretreated with phenobar- al (80 mg/kg, ip) daily for 3 successive days. The mals were used 1 day after the last injection. DP, nicotinamide, ATP, isocitric acid, and isocit- ehydrogenase (EC 1.1.1.42) were purchased from Sigma Chemical Company. (St. Louis, Mo.). $[^{45}Ca]Cl_2$ was purchased from New England Nuclear Corporation (Boston, Mass.). 1,1-Dichloroethylene was obtained from the Aldrich Chemical Co. (Milwaukee, Wis.).

Microsome preparation. After stunning, liver samples were removed and quickly homogenized (0.5 g/10 ml) in ice-cold 0.25 M sucrose containing 3 mM EDTA, pH 7. A Potter-Elvehjem homogenizer was used with the Teflon pestle driven at 750 rpm. The homogenate was centrifuged at 1500*g* for 10 min and then at 12,500*g* for 20 min. The supernatant was centrifuged for 60 min at 105,000*g*. The microsome pellet was resuspended by homogenization in ice-cold 0.1 M KCl, 0.05 M Tris buffer, pH 7.4 (TK buffer). For most experiments, the microsome suspension was used immediately after preparation. For some experiments the microsomes were quickly frozen with a dry ice ethanol bath and stored for up to 10 days at −70°C.

Incubation of 1,1-DCE or CCl_4 with liver mixed function oxidase system. Microsomes were incubated in a metabolic shaker at room temperature in tightly capped vials. Microsomes at a protein concentration of 1.0 to 1.2 mg protein/ml were incubated with a NADPH regenerating system (S) containing 100 μM NADP, 2.5 mM nicotinamide, 5 mM $MgCl_2$, 3 mM D,L-isocitrate, and 0.07 units isocitric dehydrogenase/ml in TK buffer (final volume 3 ml). 1,1-DCE or CCl_4 dissolved in ethanol, was added to the incubation medium. The maximum volume of ethanol added was 5 μliter/ml and the final concentration of 1,1-DCE or CCl_4 was 10 μliter to 1 μliter/ml. Incubation was initiated by addition of 1,1-DCE or CCl_4 to ice-cold medium.

Because of volatility of 1,1-DCE, special precautions were necessary to handle this compound. 1,1-DCE was cooled in an ice bath before use. For preincubation with the microsomal mixed function oxidase (MFO) system, cold 1,1-DCE was quickly pipetted into the preincubation mixture which was also at 0–4°C. The preincubation vial was filled to capacity with liquid, tightly capped, and finally placed in a metabolic shaker (shaking water bath) at room temperature (20–22°C).

Calcium pump activity determination. After incubation with 1,1-DCE or CCl_4 calcium pump activity was determined as previously described (11). Briefly, microsomes (0.015 to 0.05 mg/ml) were incubated in 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP (pH adjusted with imidazole to 6.8), 5 mM ammonium oxalate, 5 mM sodium azide, 20 μM $CaCl_2$, and $[^{45}Ca^{2+}]$ 0.2 μCi/ml. The assay was initiated by addition of the microsome suspension to prewarmed (37°C) assay medium. At timed intervals 0.5-ml samples were filtered through nitrocellulose filters (0.45-μm pore diameter). Filters were dried and $^{45}CaCl_2$ determined by liquid scintillation spectrophotometry. Microsomal protein was deter-

mined by the Lowery method as modified by Shatkin (12).

Lipid peroxide determination. As an index of lipid peroxidation, malonic dialdehyde was determined by a thiobarbituric acid method (13).

RESULTS

When liver microsomes were preincubated with 1,1-DCE and a NADPH generating system for 20 min, calcium uptake activity was inhibited in a concentration-dependent manner. Compared to control, calcium uptake was reduced 38% at 100 μliter/ml and inhibition reached a maximum of 55% at 1 μliter/ml (Fig. 1). No inhibition of uptake activity occurred if microsomes were incubated with as much as 1 μliter/ml 1,1-DCE in the absence of the NADPH-generating system. Ethanol at the maximal concentration used as a solvent for the chlorinated hydrocarbons did not affect calcium pump activity.

Phenobarbital pretreatment of rats has been reported to increase hepatotoxicity of a number of chlorinated hydrocarbons including 1,1-DCE. To examine whether phenobarbital pretreatment of a rat affected the *in vitro* response of the calcium pump to preincubation with 1,1-DCE we compared the effect of 1,1-DCE on calcium pump activity of liver microsomes prepared from normal rats and from phenobarbital-pretreated rats. In this study, liver microsomal calcium pump activity was inhibited 30 ± 2% in control rats and 42 ± 3% in phenobarbitol-pretreated rats when isolated microsomes were incubated with 1,1-DCE (0.3 ml/ml) for 20 min. This difference is statistically significant ($P < 0.05$ Student's t test). The current study and previous work (16) showed no difference in calcium pump activity in control and phenobarbitol-pretreated rats.

Figure 2 shows the time course of inhibition of the calcium pump after preincubation with 1,1-DCE and CCl_4 at 1 μliter/ml *in vitro*. After only 1 min of preincubation with CCl_4, calcium pump activity is inhibited almost 50%. After 5 min of preincubation, inhibition by CCl_4 reaches near maximal values of 80%. Preincubation for as long as 20 min produced only slightly greater inhibition. Preincubation with 1,1-DCE produced a substantially

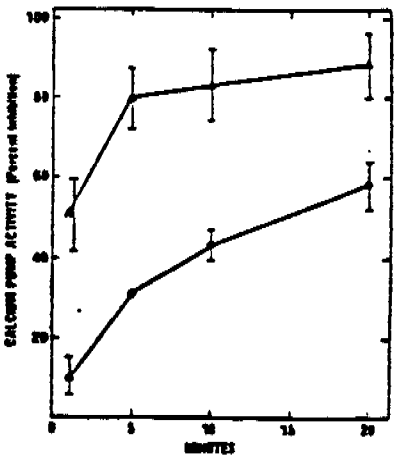

FIG. 2. Time course of liver microsome calciu pump inhibition *in vitro* by 1,1-DCE (●) and CC (▲). Liver microsomes were incubated with the ch rinated hydrocarbon (1 μliter/ml) and a NADF generating system for the time indicated in the fi ure. Samples were removed and calcium pump acti ity determined as described under Methods and M terials. Each point represents the mean ± SEM f the determination with microsomes isolated fro three animals.

different time course of calcium pump i hibition. After preincubati n for 1 mi with 1,1-DCE, activity f the calciu pump was reduced no more than 10% After 5 min, inhibition was statistical significant at 31%. Prolonging preinc bation to 20 min increased calcium pum inhibition to nearly 60%.

To confirm that 1,1-DCE did not i crease lipid peroxidation in this *in vit* system the following experiment was pe formed. Microsomes were incubated wit 1,1-DCE, CCl_4 (1 μliter/ml) or vehicle co trol in the presence of the NADPH ge erating system for 20 min at room ten perature. MDA produced during this tim period was determined. Control and 1, DCE-treated microsomes did not produ detectable MDA during this incubatio At the same concentration, incubation the presence of CCl_4 resulted in the ge eration of 0.28 ± 0.02 μg MDA/mg protei In our hands the assay could detect as li tle as the equivalent of 0.05 μg MDA/m protein measured with 1,1,3,3-tetraetho ypropane as standard. Under identical e perimental condition, CCl_4 produced

[illegible] and 1,1-DCE produced 41 ± 6% inhibition of calcium pump activity.

DISCUSSION

Disruption of calcium homeostasis has been implicated as a mediator of cell death after hepatic injury produced by either toxic chemicals or ischemia. A number of workers suggest that hepatotoxins may disrupt intracellular calcium homeostasis and there is reemphasis of the role of disrupted calcium homeostasis in cell death (14, 15). A calcium pump activity has been described in liver endoplasmic reticulum (16). This calcium pump may be analogous to the sarcoplasmic reticulum calcium pump of skeletal muscle and may participate in regulation of cytoplasmic calcium levels in liver (11, 16). This calcium pump is promptly inhibited after *in vivo* administration of a number of hepatotoxins including CCl_4 (6, 8, 9, 11), $BrCCl_3$ (8, 9), 1,1-DCE (6, 7), $CHCl_3$ (6), carbon disulfide (17), and after hepatic ischemia (10). These studies suggest that inhibition of this calcium pump may disrupt calcium homeostasis in the liver.

DCE is used in the manufacture of polyvinyl chloride products and is a potent hepatotoxin (1, 5). 1,1-DCE induced liver damage is thought to depend upon metabolic activation to a reactive metabolite by the MFO system of liver ER (18–20). Morphological studies of the early effects of 1,1-DCE demonstrate damage to the nucleus, mitochondria, and plasma membrane of hepatic cells (4). Again, in contrast to agents such as CCl_4, liver ER does not exhibit morphological changes at early times after 1,1-DCE administration (4). However, a recent study from our laboratory shows that biochemical changes occur in the liver ER at very early times after 1,1-DCE administration. Within 20 min after intraperitoneal doses of 1,1-DCE, liver ER calcium pump activity is inhibited 45% (7). This biochemical change may lead to loss of an ER calcium pool and thus represent a change of ER function (7).

If inhibition of the ER calcium pump plays a role in cell death after 1,1-DCE administration; inhibition of the pump should depend upon activation of 1,1-DCE to a reactive intermediate by the MFO system of liver ER. To demonstrate that metabolism of 1,1-DCE is essential for inhibition of the ER calcium pump, an *in vitro* system has been employed. The data presented in this report suggest that metabolism of 1,1-DCE by the MFO system is essential for calcium pump inhibition. Incubation of 1,1-DCE, an NADPH generating system and liver microsomes inhibits ER calcium pump activity. 1,1-DCE does not affect ER calcium pump activity in the absence of the NADPH generating system. This, of course, suggests that the MFO system generates a metabolite that attacks the calcium pump in liver ER. Further evidence that 1,1-DCE is metabolized before it inhibits the calcium pump is provided by enhancement of 1,1-DCE inhibition in liver microsomes from phenobarbital-pretreated rats. Phenobarbital pretreatment induces activity of the MFO system but does not affect ER calcium pump activity (11). Presumably phenobarbital pretreatment of animals results in a microsome fraction that produces reactive metabolites of 1,1-DCE to a greater extent or at a more rapid rate. Some information is available from *in vivo* studies that support these findings. 1,1-DCE treatment depletes liver glutathione and depletion of glutathione prior to 1,1-DCE administration enhances hepatotoxicity (21). Presumably glutathione serves as an endogenous substrate for conjugation of reactive 1,1-DCE metabolites. Depletion of glutathione by diethylmaleate pretreatment enhances both hepatotoxicity and calcium pump inhibition (7). Phenobarbital pretreatment has been found to produce conflicting results, such as protection of animals in some reports (4, 18) and enhancement of hepatotoxicity in others (7, 22). We have observed that both hepatotoxicity and calcium pump inhibition are enhanced *in vivo* (7).

The time course of calcium pump inhibition by CCl_4 and 1,1-DCE *in vitro* obviously differs (Fig. 2). A number of explanations can be considered. It is possible that incubation conditions are favorable for activation of CCl_4 but less so for 1,1-DCE. On the other hand, it is also possible that reactive metabolites of 1,1-DCE are

inactivated more quickly than those of CCl_4. These two possibilities have not been explored. One possible explanation has been studied. CCl_4 produces lipid peroxidation in ER membranes while 1,1-DCE does not (5, 6). Lipid peroxidation inhibits liver ER calcium pump activity (P. Ray and L. Moore, unpublished). EDTA is included in the homogenization buffer to reduce iron contamination of the microsome preparation. This minimizes NADPH-Fe^{+}-induced lipid peroxidation (23, 24). During the 20-min incubation with the NADPH generating system, lipid peroxidation is not detected in control or 1,1-DCE experiments. Incubation of these microsomes with CCl_4 and the NADPH generating system results in lipid peroxidation. This is the result of metabolism of CCl_4 to the CCl_3 free radical which in turn initiates lipid peroxidation (3, 25). The results of Waller *et al.* suggest that lipid peroxidation is not required for CCl_4 inhibition of the calcium pump (26). However, it is probable that when lipid peroxidation occurs it contributes an additional factor to calcium pump inhibition. Thus it is possible that CCl_4 inhibits the liver ER calcium pump more promptly than 1,1-DCE because lipid peroxidation contributes to pump inhibition. The difference in the ability of CCl_4 and 1,1-DCE to induce lipid peroxidation may also explain why ER damage is observed as an early morphological change after CCl_4 but not after 1,1-DCE action. Both compounds damage the ER calcium pump early after administration to an animal (7, 9, 11). But the morphological changes CCl_4 produces may be secondary to lipid peroxidation.

This study shows that 1,1-DCE inhibits the liver ER calcium pump *in vitro*. It demonstrates that *in vitro*, as well as *in vivo* (6, 7), inhibition of this pump by 1,1-DCE is not dependent upon lipid peroxidation. The work presented suggests that 1,1-DCE is activated by the MFO system to a compound that interacts with and inhibits the calcium pump.

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