Impact of Halogenated Compounds on Calcium Homeostasis in Hepatocytes

By Leon Moore,* Daniel R. Schoenberg,* and Rochelle M. Long*†

Halocarbons (CCL₄, 1,1-dichlorethylene) cause a wide spectrum of effects and injury in hepatocytes. One early effect of these compounds is the inhibition and destruction of the endoplasmic reticulum (ER) calcium pump. Subsequent to inhibition of this pump, the ER calcium pool is depleted and cytosolic levels of calcium are increased for a prolonged period of time. This effect of halocarbons has been characterized and is similar in vivo and in vitro. The importance of this redistribution of cell calcium in expression of halocarbon injury of hepatocytes has not been fully resolved. Several degradative enzymes (phospholipases, proteases) have been implicated as calcium-dependent mediators in toxicity. Our preliminary studies of the effect of calcium redistribution suggest that activation of a calcium-sensitive endonuclease in liver does not play a central role in initiating the lethal effect of halocarbons on hepatocytes.

Introduction

Cytosolic calcium is an important regulator of the activity of many metabolic and structural proteins. Cells normally maintain cytoplasmic calcium at very low levels. Calcium concentrations briefly rise several-fold in response to physiological stimuli (1). The potential role of disrupted calcium flux in chemically induced liver injury have been examined (2–6). Recently, attention has turned to examining early alterations of intracellular calcium homeostasis as causative in halocarbon-induced lethal injury (7–9).

The low resting concentration of ionized calcium in cytosol is maintained by active compartmentation processes. The level of ionized calcium in cytosol is determined by the cooperation of at least three compartmentation processes—the plasma membrane Ca²⁺-stimulated, Mg²⁺-dependent ATPase, the endoplasmic reticulum (ER) Ca²⁺-stimulated, Mg²⁺-dependent ATPase, and the mitochondrial Ca²⁺/H⁺ antiporter. Microsomal fractions isolated from a number of nonmuscle cells and tissues have an energy-dependent calcium uptake system (10–12). The calcium pump in this fraction is thought to principally represent activity of the endoplasmic reticulum (ER). Several investigators have noted similarities between these calcium pumps and the skeletal muscle sarcoplasmic reticulum calcium pump. These similarities suggest that the ER (microsomal) calcium pump functions to sequester cytoplasmic calcium and, thus, participates in regulating ionized calcium in the cytoplasm and maintaining a releasable, intracellular pool of calcium.

Reynolds and co-workers (13) first observed a selective loss of calcium from the endoplasmic reticulum of rat liver after administrations of CCL₄. Subsequently, it was demonstrated that CCL₄ rapidly causes the inhibition of the ER calcium pump (14). This pump is thought to play an essential role in regulating cytoplasmic calcium and to provide an intracellular pool of calcium in the hepatocyte that can be released in response to exogenous stimuli. When this calcium pump is destroyed, a pool of calcium appears to be released from the ER (15). More recently, we have demonstrated that cytoplasmic concentrations of ionized calcium increase rapidly and for prolonged periods both in vivo (15) and in vitro (16) after an animal or hepatocytes are exposed to CCL₄ or 1,1-dichloroethylene.

The Calcium Hypothesis

The calcium hypothesis of acute cell injury suggests that something happens during toxic injury to overload the normal homeostatic mechanisms maintaining ionized cytosolic calcium in the nanomolar range. The overload could occur by the toxicant damaging...
one or more of the energy-dependent calcium sequestration mechanisms, or by the toxicant allowing a massive influx of extracellular calcium. Halocarbons appear to inhibit or destroy either the ER or plasma membrane calcium pumps and in some cases to selectively increase membrane permeability to calcium.

A number of toxic events, including exposure to halocarbons, have been shown to elevate ionized calcium in cytoplasm to supraphysiological levels or for supraphysiological times. Currently, the element missing is demonstration of an event common to all toxicant models and resulting from calcium levels outside the normal range.

**Effect of Halocarbons on Intracellular Calcium**

**Effect on the Endoplasmic Reticulum Pump and Cytosolic Calcium**

Results from in vivo experiments are presented in Figure 1. Following a hepatotoxic, but not lethal, dose of CCl₄, the ER calcium pump activity declined to 35% of control by 30 min. Pump activity remained depressed throughout the experiment and was 6% of control by 8 hr. In livers from rats that received CCl₄, phosphorylase a activity was 133% of control by 30 min and 150% of control by 1 hr. At 8 hr hepatic phosphorylase a activity remained at 135% of control. Total phosphorylase (a + b forms) activity did not vary significantly over 8 hr in controls or in CCl₄-treated rats (data not shown). Measurement of liver cAMP concentrations demonstrated no statistically significant differences at any time between the treated and control groups (17). These observations allow us to use phosphorylase a activity as a measure of ionized calcium in rat liver cytoplasm. The activity of another endoplasmic reticulum enzyme, glucose-6-phosphatase, activity was not significantly affected until 2 hr (71% of control). By 8 hr this enzyme activity was approximately halved. In contrast, this enzyme is not inhibited after exposure of an animal to toxic doses of 1,1-dichloroethylene, a halocarbon that does not initiate lipid peroxidation (18,19). Activity of the plasma membrane enzyme 5'-nucleotidase was not affected by CCl₄ administration. To confirm the effect of CCl₄ on phosphorylase a levels, glycogen concentrations in liver were examined. Liver glycogen content was reduced to 68% of control as early as 30 min, and glycogen concentrations declined progressively until 24 hr after CCl₄, when liver glycogen could not be detected. Serum levels of glutamic-pyruvic transaminase were elevated by 8 and 24 hr to 9-fold control. The accumulation of total calcium by liver

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Time course of CCl₄-induced biochemical alterations in vivo. (A) Phosphorylase a (○), ER calcium pump (□), glucose-6-phosphates (○), and 5'-nucleotidase (■) activities in liver homogenates. (B) Glycogen (●), serum glutamic-pyruvic transaminase (○), top left scale, and liver calcium (■, top right scale) concentrations in liver or blood from the same animals. Rats were given CCl₄ (1.5 mL/kg) or vehicle at 0 min. Control and treated animals were killed at each time point (n = 3 to 6 for each group). Results are expressed as means ± SEM. In liver tissue sampled from control rats, ER calcium pump activity averaged 190 ± 11 nmole calcium/mg protein/30 min, phosphorylase a activity ranged from 44 ± 9.8 to 82 ± 11 nmole phosphate/mg protein/min at 8 AM and 5 PM, respectively; glucose-6-phosphatase activity averaged 1.59 ± 0.04 μmole phosphate/mg protein/20 min; 5'-nucleotidase averaged 0.98 ± 0.03 μmole/mg protein/20 min; glycogen content declined from 39 ± 3.4 mg/g liver to essentially zero by 24 hr because food was withheld from all animals after dosing; total liver calcium averaged 55.9 ± 1.6 μg/g liver; serum levels of glutamic-pyruvic transaminase averaged 19.6 ± 1.4 IU/L in control plasma. Differences are significant where indicated (p ≤ 0.05) by ANOVA (17).
accompanied a loss of cytoplasmic enzymes. Liver calcium rose to 207 and 382% of control levels 8 and 24 hr after CCl₄.

Results from in vitro experiments are represented in Figure 2. Cultures of rat hepatocytes (18 hr old) were exposed to a toxic concentration of CCl₄. ER calcium pump activity was depressed to 41% of control at 2.5 min, 19% of control at 5 min and pump activity remained at 10% of control after 30 min. Phosphorylase activity in cultured hepatocytes was stimulated to 472% of control at 2.5 min and to 392% of control at 5 min. Activity remained elevated beyond 30 min. Conversion of phosphorylase from the b to the a form was complete, as judged from measurement of total a + b activity (data not shown). Stimulation appeared to be greater than had been observed at early times in vivo; however, consistently low control phosphorylase a activity in vitro exaggerated this effect. Glucose-6-phosphatase activity was not significantly inhibited until 20 min after CCl₄ exposure. 5'-Nucleotidase activity was never significantly inhibited after CCl₄ exposure, as in vivo. Glycogen content of cells was depressed to 77% of control by 10 min. Glycogen continued to be mobilized at the same rate until it was 27% of control at 30 min. Later indices of liver cell injury were also examined in hepatocytes cultures. Glutamic-pyruvic transaminase release was massive by 15 and 30 min after CCl₄ (10-fold greater than control). Hepatocyte calcium (total of ionized and bound) never increased in the in vitro experiments. Only in this determination did the in vitro model depart from results observed with liver tissue from the intact rat. (See below.)

**Effect on the Plasma Membrane Pump**

Hepatotoxic doses in vivo of CCl₄, bromobenzene, or acetaminophen produce inhibition of the liver plasma membrane calcium pump (20). After CCl₄, permeability of plasma membrane vesicles to calcium is substantially increased (21). In contrast, not all hepatotoxins inhibit the ER calcium pump; bromobenzene and acetaminophen inhibit only the hepatic plasma membrane calcium pump (20).

**Effect on Mitochondrial Calcium Sequestration**

In vivo mitochondrial calcium sequestration is not inhibited by CCl₄ (14), and mitochondria accumulate great quantities of calcium parallel to tissue death, thus increasing the total calcium in the liver (13,14). In hepatocyte culture exposed to a range of CCl₄ concentrations, mitochondrial as well as ER calcium sequestration could be depressed (Fig. 3). The data presented in this figure suggest that, in our culture...
system, exposure of hepatocytes to initial CC14 concentrations than 0.1 μL/mL do not completely model CC14 action in vivo. Inhibition of mitochondrial calcium uptake by CC14 in homogenates of cultured hepatocytes accounts for the lack of calcium accumulation by the injured cells.

**Effect of Altered Calcium Homeostasis on Cell Function**

The relationship between a sustained increase of cytosolic calcium and toxicity by a number of halocarbons has lead several groups to search for a mechanism or mechanisms by which an increase of calcium could initiate an event or events ultimately leading to death of the hepatocyte. It is assumed that a prolonged increase of ionized calcium to supraphysiological levels may cause a profound and prolonged stimulation of a normal (physiological) process that irreversibly alters the plasma membrane and destroys the semipermeable character of the plasma membrane.

Recently, several groups have examined whether or not phospholipase activation may contribute to the expression of toxicity in hepatocytes. Hydrolysis of phospholipids is increased after a toxicant insult (22-25). It is widely recognized that calcium plays some role in the control of intracellular proteolysis in several tissues. This system is best characterized in skeletal muscle (26). Activation of nonlysosomal proteases in hepatocytes has been demonstrated following exposure to a toxicant (27).

**Effect of Increased Cytosolic Calcium on Endonuclease Activity**

Endonucleases are among the hepatic enzymes having activity that can be stimulated by Ca\(^{2+}\) (28,29). Activation of endonucleases has been implicated in thymocyte killing produced by glucocorticoids both in vivo (30,31) and in vitro (32). We have examined whether or not liver endonuclease activity is stimulated by this increase in intracellular ionized calcium and if such activation could play a role in initiating the ensuing hepatotoxic events.

Endonuclease activation was assessed by separating liver nuclear DNA on agarose gels to determine if DNA was fragmented. No generalized DNA fragmentation was observed in vivo, and in hepatocytes, fragmentation occurred only at very late times following the loss of plasma membrane integrity. Endonuclease activity was further examined at a more sensitive level by specifically monitoring serum albumin DNA. This gene has been shown to have characteristic cleavage sites, in the 5’ flanking region, that are hypersensitive to endonuclease action. No changes were observed in the fragment sizes of DNA prepared from rat liver or hepatocyte DNA at early times when toxicity was developing. Thus, our evidence suggests no stimulation of endonuclease activity occurs within a timeframe consistent with the rise of intracellular ionized calcium. We conclude that nucleases likely do not mediate the hepatotoxicity accompanying increased cytosolic calcium that follows halocarbon exposure.

**Summary and Conclusions**

A diversity of toxic insults, including thioacetamide, glutosamine, dimethylnitrosamine, heavy metals, and ischemia are known to alter tissue calcium content significantly. These agents may exert their effects initially by disrupting intracellular calcium homeostasis. Elevated cytosolic calcium concentrations that are supraphysiological, either in duration or in magnitude, may excessively activate a variety of calcium-dependent enzymes found at multiple locations within cells. Calcium-responsive degradative enzymes activated in an uncontrolled manner may damage sites distant (e.g., plasma membrane) from the original cellular injury. Potentially, this scheme (early redistribution of intracellular calcium, producing elevated ionized calcium in cytosol, which causes over-stimulation of calcium responsive enzymes) will prove to be a mechanism for initiating the irreversible development of cellular injury common to a wide variety of toxic substances and target tissues.

The authors thank Cynthia L. Wallace and Cynthia Cavert for their excellent technical assistance. This work was supported by grant ES03437 from the National Institute of Environmental Health Sciences, NIH, Public Health Service.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting
the views of the Department of Defense or USUHS. These experiments were conducted according to the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, DHHS Pub. No. (NIH) 78-23.

REFERENCES

1. Carafoli, E. Membrane transport and the regulation of the cell calcium levels. In: Pathophysiology of Shock, Anoxia, and Ischemia (R. A. Cowley and B. F. Trump, Eds.), The Williams and Wilkins Company, Baltimore, 1982, pp. 95–112.

2. Schanne, F. A. X., Kane, A. B., Young, E. A., and Farber, J. L. Calcium dependence of toxic cell death: a final common pathway. Science 205: 700–702 (1979).

3. Casini, A. F., and Farber, J. L. Dependence of the CCl4-induced death of cultured hepatocytes on the extracellular calcium concentration. Am. J. Pathol. 105: 138–148 (1981).

4. Smith, M. T., Thor, H., and Orrenius, S. Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. Science 213: 1257–1259 (1981).

5. Chenery, R. Geroge, M., and Krishna, G. The effect of ionophore A23187 and calcium on CCl4-induced toxicity in cultured hepatocytes. Toxicol. Appl. Pharmacol. 50: 241–252 (1981).

6. Fariss, F. W., Pascoe, G. A., and Reed, J. D. Vitamin E reversal of the effect of extracellular calcium on chemically-induced toxicity in hepatocytes. Science 227: 751–754 (1985).

7. Recknagel, R. O. A new direction in the study of CCl4 hepatotoxicity. Life Sci. 33: 401–408 (1983).

8. Pencil, S. D., Brattin, W. J., Glende, E. A., and Recknagel, R. O. Evidence against involvement of calcium in CCl4-dependent inhibition of lipid secretion by isolated hepatocytes. Biochem. Pharmacol. 33: 2425–2429 (1984).

9. Bellomo, G., Thor, H., and Orrenius, S. Increase in cytosolic Ca2+ concentrations during t-butyl hydroperoxide metabolism by isolated hepatocytes involves NADPH oxidation and mobilization of intracellular Ca2+ stores. FEBS Lett. 168: 38–42 (1984).

10. Moore, L., Chen, T., Knapp, Jr., H. R., and Landon, E. J. Energy-dependent calcium sequestration activity in rat liver microsomes. J. Biol. Chem. 250: 4563–4568 (1975).

11. Moore, L. and Pastan, I. Energy-dependent calcium uptake activity in cultured mouse fibroblast microsomes. Regulation of the uptake system by cell density. J. Biol. Chem. 252: 6304–6309 (1977).

12. Burns, D. E., McDonald, J. M., and Jarett, L. J. Energy-dependent calcium transport in endoplasmic reticulum of adenocytes. Biol. Chem. 251: 7191–7197 (1976).

13. Reynolds, E. S., Ree, H. J., and Moslen, M. T. Liver parenchymal cell injury. IX. Phenobarbital potentiation of endoplasmic reticulum denaturation following CCl4. Lab Invest. 26: 290–296 (1972).

14. Moore, L., Davenport, G. R., and Landon, E. J. Calcium uptake of a rat liver subcellular fraction in response to in vivo administration of CCl4. J. Biol. Chem. 251: 1197–1201 (1976).

15. Long, R. M., and Moore, L. Inhibition of liver endoplasmic reticulum calcium pump by CCl4 and release of a sequestered calcium pool. Biochem. Pharmacol. 35: 4131–4137 (1986).

16. Long, R. M., and Moore, L. Elevated cytosolic calcium in rat hepatocytes exposed to carbon tetrachloride. J. Pharmacol. Exp. Ther. 238: 196–191 (1986).

17. Long, R. M., and Moore, L. Biochemical evaluation of rat hepatocyte primary cultures as a model for carbon tetrachloride hepatotoxicity: comparative studies in vivo and in vitro. Toxicol. Appl. Pharmacol. 92: 295–306 (1988).

18. Jaeger, R. J., Trabulus, M. J., and Murphy, S. D. Biochemical effects of 1,1-dichloroethylene in rats: dissociation of its hepatotoxicity from a lipoperoxidative mechanism. Toxicol. Appl. Pharmacol. 24: 457–467 (1978).

19. Moore, L. Inhibition of liver microsomal calcium pump by in vivo administration of CCl4, CHCl3 and 1,1-dichloroethylene (vinylidene chloride). Biochem. Pharmacol 29: 2505–2511 (1980).

20. Tsokos-Kuhn, J. O., Todd, E. L., McMillin-Wood, J. B., and Mitchell, J. R. ATP-dependent calcium uptake by rat liver plasma membrane vesicles: effect of alkylating hepatotoxins in vivo. Mol. Pharmacol. 28: 56–61 (1985).

21. Tsokos-Kuhn, J. O., Smith, C. V., Mitchell, J. R., Tate, C. A., and Entman, M. L. Evidence for increased membrane permeability of plasmalemmal vesicles from livers of phenobarbital-induced CCl4-intoxicated rats. Mol. Pharmacol. 30: 444–451 (1986).

22. Chien, K. R., Abrams, J., Serroni, A., Martin, J. T., and Farber, J. L. Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischemic liver cell injury. J. Biol. Chem. 2253: 4809–4817 (1978).

23. Farber, J. L., and Young, E. E. Accelerated phospholipid degradation in anoxic rat hepatocytes. Arch Biochem. Biophys. 211: 312–320 (1981).

24. Lamb, R. G., McCue, S. B., Taylor, D. R., and McGuffin, M. A. The role of phospholipid metabolism in bromobenzene-and carbon tetrachloride-dependent hepatocyte injury. Toxicol. Appl. Pharmacol. 74: 510–520.

25. Glende, E. A., Jr., and Pushpendran, C. K. Activation of phospholipase A2 by carbon tetrachloride in isolated rat hepatocytes. Biochem. Pharmacol. 35: 3301–3307 (1986).

26. Ishiura, S. Calcium-dependent proteolysis in living cells. Life Sci. 29: 1079–1087 (1981).

27. Nicotera, P., Hartzeil, P., Baldi, C., Svensson, S-A, Bellomo, G., and Orrenius, S. Cystamine induces toxicity in hepatocytes through the elevation of cytosolic Ca2+ and the stimulation of a non-lysosomal proteolytic system. J. Biol. Chem. 261: 14628–14635 (1986).

28. Burgoine, L. A., and Mobbs, J. The reaction of the Ca-Mg endonuclease with the A-sites of rat nucleoprotein. Nucleic Acids Res. 2: 1551–1558 (1975).

29. Hewish, D. R., and Burgoine, L. A. Chromatin substructure. The digestion of chromatin DNA at regularly spaced sites by nuclear deoxyribonuclease. Biochem. Biophys. Res. Commun. 52: 475–481 (1978).

30. Wylie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555–556 (1980).

31. Compton, M. M., and Cidlowski, J. A. Rapid in vivo effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. Endocrinology 118: 38–45 (1986).

32. Cohen, J. J., and Duke, R. C. Glucocorticoid activation of a calcium-dependent endonuclease in thymic nuclei leads to cell death. J. Immunol. 132: 38–42 (1984).