Interactive Toxicity and Stress Protein Expression by Vinlylidene Chloride and Monochloroacetate in Precision-Cut Rat Liver Slices

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Vinylidene chloride (VDC) is a groundwater and drinking water contaminant. Monochloroacetic acid (MCA) is a chlorination by-product of drinking water. Because environmental or occupational exposure to chemicals takes place at low concentrations, a sensitive in vitro system of liver slices was used to examine the interactive toxicity of MCA and VDC. Liver slices from Sprague-Dawley rats were exposed to 100 μM MCA for 1 hr before exposure to 20 or 48 μM VDC and incubated for 1 to 8 hr. MCA + 48 μM VDC resulted in a significant leakage of K* by 4 hr, while MCA + 20 μM VDC did not. At 4 hr, MCA + 48 μM VDC resulted in centrilobular necrosis. MCA caused a significant depletion of slice glutathione (GSH) at 1 hr, which was maintained up to 3 hr. As reactive VDC metabolites are detoxified by conjugation with GSH, the increase in VDC toxicity by MCA is possibly due to GSH-depleting effects of MCA. Heat shock protein (HSP) 72 was increased 2.5-fold by MCA + 20 μM VDC as early as 2 hr, although K* leakage was not increased. MCA + 48 μM VDC resulted in a 3-fold increase in HSP 72 by 2 hr, while there were modest increases in HSPs 60 and 32. Therefore, HSP 72 is an early sensitive indicator of interactive toxicity of nontoxic concentrations of MCA and VDC. This is the first time that micromolar concentrations of these drinking water contaminants were observed to affect cellular homeostasis in the liver. — Environ Health Perspect 106(Suppl 6):1319-1323 (1998).
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Coexposure to environmental pollutants is a common concern, particularly if the pollutants can affect each other’s toxicity. Vinylidene chloride (VDC) is used in industry for the manufacture of Saran-type (Johnson Wax, Racine, Wisconsin) plastics and as a degrading agent. It occurs as a contaminant in the groundwater, with groundwater contamination reaching 21,000 μg/l or > 200 μM in Arizona (1). VDC has been identified as a contaminant in drinking water. It is ranked eleventh among the hazardous chlorinated organic compounds found in drinking water in this country (2). Approximately 50% of the population of the United States relies on groundwater as a source of drinking water. The major method of drinking water disinfection in the United States is by chlorination (3). Monochloroacetic acid (MCA) is formed as a result of chlorination of drinking water for disinfection and can be present at concentrations of approximately 1 μg/l (4). Therefore, there is possible coexposure of humans to these chemicals.

Both of these environmental pollutants are hepatotoxic and interact with each other. VDC causes centrilobular necrosis of the liver and the elevation of serum enzymes, indicating hepatocellular damage (5–7). Although VDC was a rodent carcinogen in chronic bioassays (8–10), MCA was not. However, MCA is more toxic than di- or trichloroacetic acids in acute toxicity studies. Exposure of mouse hepatocytes to 5 mM MCA resulted in an increased enzyme leakage, whereas 5 mM di- or trichloroacetic acids had no effect (11). Pretreatment of rats with MCA 1 hr earlier resulted in a significant increase in VDC hepatotoxicity (12).

Because environmental exposure to MCA or VDC takes place at low levels, this study was undertaken to examine the interactive toxicity of subtoxic concentrations of MCA and VDC in precision-cut rat liver slices and the mechanism of MCA and VDC interaction. We previously demonstrated that stress proteins can be used as early sensitive indicators of toxicity of subtoxic concentrations of chemicals such as sodium arsenite in precision-cut rat liver slices (13). Therefore, expression of stress proteins by subtoxic concentrations of MCA and VDC was also studied to examine whether they could be used as early sensitive indicators of interactive toxicity of these drinking water contaminants.

Materials and Methods

Male Sprague-Dawley rats (275–350 g) were used in these experiments. Liver slices (250–300 microns) were prepared and incubated by previously described methods (13). Toxicants were added after a 1-hr preincubation period. MCA (100 μM; Sigma, St. Louis, Missouri) was dissolved directly in the medium and the pH was adjusted to 7.4. VDC (Aldrich, Milwaukee, Wisconsin) was added 1 hr after incubation with MCA. The amounts of VDC used were 0.05 or 0.1 μl, which resulted in medium concentrations of 20 and 48 μM, respectively (14). VDC was mixed with dimethyl sulfoxide (DMSO) to obtain the appropriate concentrations and was injected onto a paper wick suspended inside the incubation vial. No addition was made to the control slices, as previous studies have shown that only minute amounts of DMSO leave the paper wick and no toxic effects were seen (14). Liver slices were incubated 1 to 8 hr and one slice was placed in 1 ml deionized water for the determination of K+/DNA (15). One slice was placed in 1 ml 10% TCA for slice glutathione (GSH) assay (16). Two slices were pooled for the Western blot analyses of heat shock proteins (HSPs). For histologic examinations, liver slices were fixed in 10% neutral buffered formalin, embedded in paraffin wax, processed for light microscopy, and stained with hematoxylin and cosin.

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Abbreviations used: DMSO, dimethyl sulfoxide; GSH, glutathione; HSP, heat shock protein; MCA, monochloroacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; VAC, cytoplasmic vacuolations; VDC, vinylidene chloride.
Western Blot Analysis
Liver slices were preincubated with MCA for 1 hr and exposed to VDC for a further 2 hr. Slice homogenate was centrifuged at 10,000 x g for 20 min and the supernatant fraction was used for the analysis. Protein (40 µg) was loaded onto a 10 or 12% gel, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred onto a polyvinylidene difluoride paper. Immunoperoxidase staining for HSPs 72, 60, 32, and 90 was performed using specific monoclonal antibodies for the inducible forms (StressGen, Victoria, British Columbia, Canada), peroxidase conjugated secondary antibodies, and 3,3′-diaminobenzidine tetrahydrochloride. Densitometric analysis of the bands was performed using the Bio Image Whole Band Analysis 3.2.1. program (Bio Image, Ann Arbor, Michigan).

Determination of Slice Glutathione
The slice GSH contents were determined as nonprotein sulphydryl according to Ellman (16), with modifications suitable for liver slices. After incubation, slices were washed twice in slicing buffer, placed in 1 ml 10% TCA, and sonicated. The resulting homogenate was centrifuged for 20 min at 10,000 rpm. To 125 µl of the supernatant, 375 µl dH2O was added and vortexed. To this, 2 ml Na2HPO4 (300 mM) and 0.5 ml 5,5′-dithio-bis-2-nitrobenzoic acid (1 mM)/sodium citrate (340 mM) were added and vortexed. The absorbance was measured at 412 nm using a Beckman (Beckman Instruments, Fullerton, California) DU7 spectrophotometer. A standard curve was prepared using GSH. The protein content of the pellet was determined by the BCA (Bicinchoninic Acid Protein Assay Kit, Sigma) method. The slice GSH is expressed as nmole GSH/milligram protein.

Statistics
Results for K+/DNA and GSH were analyzed using analysis of variance, and the means were compared by Dunn’s multiple comparison test, n = 3. p < 0.05 was taken as significant. Four liver slices were used at each time point and for each condition for the K+/DNA analysis and two liver slices were used for GSH analysis and for histologic analysis. Two liver slices were pooled for Western blot analysis.

Results
Interactive Toxicity of Monochloroacetic Acid and Vinylidene Chloride
At 4 and 8 hr, MCA + 8 µM VDC resulted in a significant leakage of intracellular K+, while MCA + 20 µM VDC did not cause any leakage of K+ (Figure 1).

Histologic Analysis of Liver Slices
The histologic changes in liver slices are summarized in Table 1. MCA alone did not result in any changes in liver slices at any time (Figure 2B), as compared to control slices (Figure 2A). Twenty-micromolar VDC caused cytoplasmic vacuolations in hepatocytes (Figure 2C) and no necrotic changes up to 6 hr. MCA + 20 µM VDC resulted in an increase in the amount of vacuolation at 2 hr and minimal necrotic changes (+1) by 4 hr (Figure 2D). Forty-eight-micromolar VDC resulted in minimal necrotic changes at 4 and 6 hr, while MCA + 48 µM VDC (Figure 2E) caused moderate necrosis at 4 hr and marked necrosis at 6 hr.

Effects of Monochloroacetic Acid on Glutathione Contents of Rat Liver Slices
Monochloroacetic acid resulted in a significant depletion of GSH by 1 hr (37%), which was maintained up to 3 hr. At 2 and 3 hr, the depletion of GSH by MCA was 39 and 45%, respectively (Figure 3). The decrease in liver slice GSH content was not further enhanced by the presence of VDC (Figure 4).

Expression of Stress Proteins by Monochloroacetic Acid and Vinylidene Chloride in Rat Liver Slices
A representative Western blot analysis for HSP 72 is shown in Figure 5. MCA alone did not cause an increase in HSP 72 contents at 2 hr (Figure 6). VDC alone caused a 30% increase in HSP 72 levels. MCA + 20 µM VDC resulted in a 2.5-fold increase in HSP 72 contents as compared to control, while MCA + 48 µM VDC resulted in a 3-fold increase in HSP 72. The increases in the amounts of HSP 60, HSP 32, and HSP 90 were lower than that observed for HSP 72 due to the combined treatments of MCA and VDC (Table 2).

Discussion
Previous studies have demonstrated that MCA pretreatment increases the hepatotoxicity of chloroform in rats (17). Earlier studies have shown that VDC hepatotoxicity can be manipulated by altering P450 activity and glutathione levels but these were performed at hepatotoxic doses of VDC (18,19). In this study liver slices were exposed to subtoxic concentrations of MCA and VDC to examine whether low concentrations of these chemicals can interact to cause damage to liver slices. The concentration of VDC and MCA used in these in vitro studies exceeds the quantities seen in groundwater or drinking water but are at subcytotoxic levels. Thus the effects seen may be indicative of those expected with longer exposures to low levels of these toxicants.

Pretreatment of liver slices with 100 µM MCA increased the toxicity of 48 µM VDC.
Figure 2. Histologic analysis of liver slices. (A) Control liver slice at 2 hr. Liver slice treated with (B) 100 μM MCA for 2 hr; (C) 20 μM VDC for 2 hr, showing fine cytoplasmic vacuolations (arrow); (D) MCA + 48 μM VDC for 2 hr, showing minimal (+1) centrilobular necrosis (arrow points to the edge of the necrotic lesion); and (E) MCA + 48 μM VDC, showing moderate centrilobular necrosis (+3) at 4 hr. Magnification, x80.
by 4 hr. No increase in K+ leakage was observed with MCA+20 μM VDC. VDC treatment results in the centrilobular necrosis of the liver in fasted rats (20). Histologic analysis indicated that MCA pretreatment resulted in an increase in the minimal necrotic lesions caused by VDC alone to moderate or marked centrilobular necrosis in 48-μM VDC-exposed liver slices.

VDC is bioactivated by cytochrome P450IIE1 in the liver (27) to reactive metabolites chloroacetyl chloride and dichloroacetaldehyde. Chloroacetyl chloride is hydrolyzed to form MCA (22).

These reactive VDC metabolites can bind to hepatic macromolecules and cause toxicity (23). The major pathway for the detoxication of reactive VDC metabolites is by conjugation with GSH (24). Fasting and other conditions that decrease hepatic GSH enhance the injury caused by VDC (25,26). Because MCA caused a significant depletion of GSH at the time when VDC was introduced into the incubation system, the reactive VDC metabolites may bind to hepatocellular macromolecules, resulting in damage to liver slices.

VDC alone resulted in a modest increase in HSP 72 at 2 hr, although it did not cause an increase in K+ leakage. Although MCA+20 μM VDC or MCA+48 μM VDC did not result in a leakage of slice K+ at 2 hr, these treatments resulted in a marked increase in HSP 72 levels at this time. The combined treatments of MCA and VDC also resulted in smaller increases in HSP 60, HSP 32, and HSP 90 contents. Therefore, HSP 72 is an early sensitive indicator of interactive hepatotoxicity of MCA and VDC.

In normal physiological states of cells, stress proteins occur at very low levels. When exposed to unfavorable conditions such as hypothermia, xenobiotics, ultraviolet light, anoxia, oxidizing agents, or heavy metals, there is a considerable increase in the expression of stress proteins (27–29). Enhanced synthesis of stress proteins has been proposed as a biomarker for toxicity because there is a relationship between the individual levels of these proteins and the degree of cellular damage inflicted (30–32). The accumulation of denatured proteins results in an increase in the synthesis of stress proteins (33). The mechanism of toxicity of VDC is due to the covalent modification of critical target proteins by its reactive metabolites including MCA (23). The protein denaturing effect of these VDC metabolites probably acts as a signal for the increased synthesis of HSP 72, which is the major cytosolic stress protein induced by a variety of stressors (27). Levels of HSP 90, another cytosolic stress protein (27), is not altered much by MCA+VDC treatments. HSP 32 is heme oxygenase 1, which is an antioxidant enzyme that is induced under conditions of oxidative stress (34). HSP 60 is a mitochondrial stress protein (35). VDC causes mitochondrial damage in hepatocytes (36). In the present experiments the low concentrations of VDC used probably resulted in modest increases in HSP 32 and HSP 60.

Table 2. Expression of stress proteins after monochloroacetic acid and vinylidene chloride treatment.*

| HSP expressed | MCA | 20 μM VDC | 48 μM VDC | MCA+20 μM VDC | MCA+48 μM VDC |
|---------------|-----|-----------|-----------|---------------|---------------|
| 72            | 0%  | 70%       | 63%       | 2.5-fold      | 3-fold        |
| 60            | 0%  | 10%       | 21%       | 38%           | 39%           |
| 32            | 0%  | 0%        | 0%        | 34%           | 82%           |
| 90            | 0%  | 22%       | 14%       | 42%           | 26%           |

*Liver slices were preincubated with 100 μM MCA and exposed to 20 or 48 μM VDC for 2 hr. Expression of specific stress proteins was quantified by densitometric analysis of the Western blot analyses. Values are expressed as percent increase or -fold increase as compared to control.
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