Vitamin A Potentiation of Vinylidene Chloride Hepatotoxicity in Rats and Precision-Cut Rat Liver Slices

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Pretreatment of large doses of vitamin A (VA) is known to potentiate the hepatotoxicity of carbon tetrachloride. Therefore the effects of 1-day VA pretreatment on VDC hepatotoxicity was examined both in vivo and in an in vitro system of precision-cut rat liver slices. Male Sprague–Dawley rats were pretreated with 250,000 IU/kg VA by oral gavage. After 24 hr rats were administered 50, 100, or 200 mg/kg VDC ip. Precision-cut liver slices were prepared from VA pretreated rats 24 hr later and the liver slices were exposed for 2–8 hr to 0.025–1.0 μl VDC evaporated into the gas phase of the incubation vials. VA pretreatment resulted in an enhancement of VDC toxicity, both in vivo and in vitro. There was a dose-dependent increase in plasma ALT 24 hr after VDC treatment of rats and an increase in K⁺ leakage from liver slices after VDC exposure. Histological analysis of the liver or the liver slices revealed that VA + VDC treatment resulted in centrilobular necrosis of the liver. When GdCl₃ (10 mg/kg iv) was administered just before VA pretreatment of rats, VDC toxicity was partially reversed as observed by a decrease in ALT in vivo and a decrease in the loss of K⁺ in vitro. These results indicated that Kupffer cells, the resident macrophages of the liver, were partially responsible for the VA-potentiated VDC hepatotoxicity. One-day pretreatment of VA induced cytochrome P450IIE1 protein content as well as its enzymatic activity as measured by p-nitrophenol hydroxylate. Because VDC is bioactivated by cytochrome P450IIE1, the increase in VDC hepatotoxicity after VA may be due to an increased bioactivation of VDC in the liver and in precision-cut liver slices. Thus, more than one mechanism may be involved in the VA enhancement of VDC hepatotoxicity.

Vitamin A (VA, retinol) is an essential nutrient found naturally in several foods. VA supports several functions in the body such as vision, growth, reproduction, and maintenance and differentiation of epithelial tissues. Over-the-counter VA supplements are often used for general health promotion and prophylaxis against common cold. It is also used therapeutically for the treatment of skin conditions such as acne and psoriasis, and for cancer chemoprevention. About 35–50% of adults in the United States regularly consume vitamin A supplements (McDonald, 1986). Acute or chronic intake of large doses of VA can result in hepatotoxicity. Even therapeutic doses of VA taken for 2–15 years have been shown to produce liver damage. In patients who had chronic hypervitaminosis A, the mean daily intake of VA in patients who had cirrhosis was 135,000 IU/day, while that in patients with noncirrhotic liver disease was 66,000 IU/day (Guebel et al., 1991). There is a recent report that indicates that even lower daily, over-the-counter VA supplements can cause liver disease. For example, intake of 25,000 IU/day supplement resulted in reversible hepatotoxicity (Kowalski et al., 1994).

Vitamin A toxicity can be modulated by simultaneous exposure to environmental chemicals, drugs, food substances, and other dietary supplements. VA pretreatment for 7 days potentiated the hepatotoxicity of CCl₄ (ElSisi et al., 1993a,b), allyl alcohol, acetaminophen, and endotoxin (El-Sisi et al., 1993a). Recent investigations have demonstrated that 1-day pretreatment of VA also resulted in the enhancement of CCl₄ hepatotoxicity in rats (Badger et al., 1995). VA also increases ethanol hepatotoxicity in rats (Leo et al., 1982). Simultaneous exposure of rats to a nonhepatotoxic level of VA and ethanol for 9 months resulted in liver damage as indicated by an increase in liver enzymes in the serum as well as morphologic changes such as cellular infiltration, loss of hepatocytes, mitochondriol damage in hepatocytes, and fibrosis (Anonymous, 1983). The mechanism by which VA increased the toxicity of the chemicals discussed above is not fully known. However, VA is thought to prime Kupffer cells (Mobley et al., 1991) in such a way that treatment with a hepatotoxicant results in a greater release of cytotoxic mediators by the VA-primed Kupffer cells resulting in greater damage. Kupffer cells, the resident macrophages of the liver, may play a role in the potentiation of CCl₄-induced toxicity. It has been shown that the inactivation of Kupffer cells by treatment with gadolinium chloride reduces CCl₄-induced liver injury (Edwards et al., 1993). Gadolinium chloride causes disintegration of Kupffer cells, thereby caus-
TABLE 1

<table>
<thead>
<tr>
<th>Volume of VDC injected (µl)</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDC concentration in media (µM)</td>
<td>12</td>
<td>20</td>
<td>48</td>
<td>170</td>
<td>340</td>
<td>708</td>
</tr>
</tbody>
</table>

Note. Different quantities of VDC dissolved in DMSO were injected onto a paper wick suspended inside vials containing 1.7 ml Waymouth's medium and incubated for 30 min or 1 or 2 hr. An aliquot of the medium was analyzed by gas chromatography as described under Materials and Methods. VDC concentrations in the media are shown as the means of the concentrations at different times of incubation (n = 6).

Vinylidene chloride (VDC) is a chlorinated hydrocarbon which is used as a degreasing agent and for the manufacture of Saran-type plastics. VDC causes hepatotoxicity in laboratory rodents (Jaeger et al., 1974; Andersen et al., 1979; Andersen and Jenkins, 1977; Reynolds et al., 1980; Jenkins and Andersen, 1978). It is present as a contaminant in the ground water (Office of Technology Assessment, 1984). In some parts of the country, the amounts of VDC in ground water far exceed the maximum contaminant level (MCL) for VDC set by the EPA, which is 7 ppb. For example, concentrations up to 21,000 µg/liter VDC (21 ppm) have been reported in Arizona (Graf, 1986). Because 50% of the U.S. population relies on ground water as the source of drinking water, there is a potential for the coexposure of VA and VDC. VDC has been identified as a contaminant in chlorinated drinking water and has been ranked 11th in the United States as a hazardous chlorinated organic substance in drinking water (Deinzer et al., 1978). VDC has been reported in the tap water from several locations in this country (Eurocop-Cost, 1976; Wallace et al., 1986).

The present study was undertaken in order to examine whether VA pretreatment enhances VDC hepatotoxicity using an in vitro system of precision-cut rat liver slices, and also in vivo, and to determine the mechanisms responsible for this potentiation. The purpose of this study is to examine whether VA increases VDC hepatotoxicity and, if so, the role of Kupffer cells and cytochrome P450IIIE1 in the VA enhancement of VDC hepatotoxicity.

MATERIALS AND METHODS

Materials. VDC and GdCl₃ were purchased from Aldrich (Milwaukee, WI). Vitamin A (retinol) was obtained as Aquasol from Armour Pharmaceutical (Kankakee, IL). Vehicle control for VA consisted of water, propylene glycol, and Tween 20 in the ratio of 83:10:7.

Animals. Male Sprague-Dawley rats (250-275 g) were purchased from Harlen (Indianapolis, IN). The animals were allowed to acclimatize for 1 week before use. Food and water were freely available to the rats.

In vivo studies. To determine the effects of VA pretreatment on VDC hepatotoxicity, rats were administered 250,000 IU/kg (75 mg/kg) VA by

![FIG. 1. Cytotoxicity of VDC in rat liver slices. One hour after preincubation liver slices were exposed to 0.025–1.0 µl VDC for 2, 4, or 8 hr. Control slices were incubated without VDC. * indicates significantly different from the control.](image1)

![FIG. 2. Effects of VA pretreatment on VDC cytotoxicity in rat liver slices. Rats were pretreated with VA (250,000 IU/kg) or the vehicle by gavage 24 hr before preparation of liver slices. One hour after preincubation liver slices were exposed to 0.025–0.10 µl VDC for 2, 4, or 8 hr. Control slices were incubated without VDC. * indicates significantly different from the vehicle control. # indicates significantly different from VA alone.](image2)
To examine the role of Kupffer cells in the VA potentiation of VDC toxicity, rats were pretreated with GdCl$_3$ (as described in in vivo studies) just before VA was given on Day 1. Rats were euthanized on Day 2. The liver was excised and placed in ice-cold Kreb's Hensleit buffer.

Liver slices were prepared as previously described (Wijeweera et al., 1995). Briefly, liver slices (250–300 μm) were prepared from cylindrical cores of liver tissue of 10 mm diameter, using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL). Liver slices were stored in cold Kreb's Hensleit buffer that had been gassed with 95% O$_2$/5% CO$_2$ until use. Two liver slices were loaded onto a semicircular stainless-steel screen with two steel rings at each end and the screen was placed in a glass scintillation vial containing 1.7 ml Waymouth MB751/2 to which 0.5% gentamycin has been added and gassed with 95% O$_2$/5% CO$_2$ for 30 min before use. The vials were gassed with 95% O$_2$/5% CO$_2$ and incubated at 37°C on a roller incubator as previously described (Smith et al., 1986). Liver slices from control or treated rats were exposed to different concentrations of VDC for 2, 4, or 8 hr after 1 hr preincubation.

Two series of experiments were performed with different amounts of VDC. VDC was evaporated into the head space (22 ml) of the incubation vial. One set of experiments the volumes of VDC used were 0.25, 0.5, or 1.0 μl. In the other set, the amounts of VDC used were 0.025, 0.05, or 1.0 μl. VDC was diluted in DMSO to obtain proper concentrations and injected onto a paper wick mounted on a pin passing through the septum fitted to the cap of the vial. Exposure to DMSO alone did not affect the slice viability. Slice viability was assessed by the measurement of the leakage of intracellular K$^+$ across the capillary membrane to the media was measured by gas chromatography. VDC mixed with DMSO was injected onto a paper wick suspended inside the vial containing 1.7 ml Waymouth's medium and gassed for 0.5, 1, or 2 hr. An aliquot of 0.25 ml of the media was removed from vials by a gas-tight syringe and placed in a glass scintillation vial through a septum in the cap of the vial. The vials were gassed with 95% O$_2$/5% CO$_2$ for 30 min. An aliquot of the gas phase (0.25 ml) was injected into a 6 ft x ½ in. Poropack-QS 80/100 column (Supelco, Bellefonte, PA) on a HP5890 gas chromatograph with flame ionization detection. The limit of detection was 0.5 nm. The experiment was repeated three times with two samples for each condition.

### TABLE 2
Grading of Centrilobular Necrosis of Liver Slices from Vehicle (C) or VA-Pretreated Rats Exposed to VDC

<table>
<thead>
<tr>
<th>VDC (μl):</th>
<th>0</th>
<th>0.025</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hr): 2</td>
<td>C</td>
<td>VA</td>
<td>C</td>
<td>VA</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>VA</td>
<td>C</td>
<td>VA</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>VA</td>
<td>C</td>
<td>VA</td>
</tr>
<tr>
<td>Pretreatment: C</td>
<td>VA</td>
<td>C</td>
<td>VA</td>
<td>C</td>
</tr>
</tbody>
</table>

**Grading:***

- 0 (normal)
- 1 (minimal)
- 2 (mild)
- 3 (moderate)
- 4 (marked)
- 5 (severe)

*Note.* Rats were pretreated with VA (250,000 IU/kg) 24 hr before preparation of liver slices. Liver slices were exposed to 0.025–1.0 μl VDC for 2, 4, or 8 hr. Control slices were incubated without VDC. Liver slices were fixed in 10% Formalin, processed for light microscopy, and stained with H&E (n = 3, two liver slices from each rat were used for each condition).
FIG. 4. Histopathology of liver slices from VA-pretreated rats exposed to VDC. Rats were pretreated with 250,000 IU VA po 24 hr before preparation of liver slices. Controls received the vehicle. After 1 hr preincubation liver slices were exposed to 0.025–0.1 μl VDC for 2, 4, or 8 hr. (A) VA control. (B) VA + 0.05 μl VDC at 2 hr showing minimal necrotic changes and vacuolar changes in the midzonal hepatocytes. (C) VA + 0.025 μl VDC at 4 hr showing mild centrilobular necrosis. Arrow points to necrotic cells. (D) VA + 0.05 μl VDC at 4 hr showing moderate centrilobular necrosis. (E) VA + 0.05 μl VDC at 8 hr showing severe centrilobular necrosis. Original magnification, ×100.

Effects of VA on cytochrome P450 activity as detected by PNP hydroxylation by liver slices. PNP hydroxylation was performed according to methods described previously (Koop, 1986; Reinke and Moyer, 1985), with a few modifications. Liver slices were prepared from VA- or vehicle-pretreated or cytochrome P450IIIE1-induced rats by treatment with 100 mg/kg pyridine ip 24 hr earlier (Kim and Novak, 1990). Twenty slices from each rat were blotted and placed in a 25-ml Erlenmyer flask with a raised bottom, containing 5 ml RPMI 1640 medium (pH 7.4) without phenol red (Gibco BRL, Grand Island, NY) to which 100 μM p-nitrophenol (Calbiochem, San Diego, CA) has been added. Twenty slices were used to provide enough biotransformation activity for this assay. The flasks were incubated at 37°C for 15 min in an orbital shaker (75 rpm). Flasks were placed on ice after incubation. Slices were blotted and placed in 2 ml deionized water and homogenized by sonication. The medium was decanted into a test tube,
Control rats received the vehicle treatments. Twenty-four hours after VDC, rats were treated with 100 mg/kg DAS in 1 ml corn oil (CO) ip. In the VA potentiation of VDC hepatotoxicity, rats were pretreated with Diallyl sulfide (DAS) is a known inhibitor of cytochrome P450IIE1 protein. Therefore, in order to investigate the role of cytochrome P450IIE1 protein in the VA potentiation of VDC hepatoxicity, rats were pretreated with VA 24 hr before the administration of 50 mg/kg VDC. Four hours before VDC, rats were treated with 100 mg/kg DAS in 1 ml corn oil (CO) ip. Control rats received the vehicle treatments. Twenty-four hours after VDC, rats were euthanized by an overdose of pentobarbital, and blood was collected from the abdominal aorta for the determination of plasma ALT.

Statistical analysis. Results were analyzed using ANOVA and the means were compared by Dunn's multiple comparison test. p < 0.05 was taken as significant. For the in vivo study, n = 4–5. For the in vitro study, n = 3–4. Four liver slices were used at each time point and for each condition.

FIG. 5. Plasma ALT levels of rats exposed to VDC. Rats were pretreated with VA or GdCl3 + VA, 24 hr before 50, 100, or 200 mg/kg VDC. Control rats received the vehicle. * indicates significantly different from vehicle +50 mg/kg VDC. ** indicates significantly different from VA + 50 mg/kg VDC. (Error bars are not shown for treatment groups 4 and 7 because one animal survived in each group. For treatment groups 1, 2, 9, and 11 the error bars are too small.)

VITAMIN A POTENTIATION OF VDC HEPATOTOXICITY

Results

VDC Concentrations in the Media

The VDC concentration in the media was analyzed 0.5, 1, and 2 hr after incubation. The VDC in the media remained constant up to 2 hr with little variability (Table 1). For the ease of presentation, the quantities of VDC used in the in vitro experiments will be presented as the volumes injected into the vials.

Effects of VDC on Liver Slices

At 8 hr, 0.1–1.0 μl VDC resulted in a significant decrease in slice viability (Fig. 1). Exposure to VDC quantities of 0.025–0.05 μl VDC did not have a statistically significant effect on the slice K+ contents.

Effects of VA Pretreatment on VDC Toxicity in Liver Slices

VA pretreatment significantly increased the toxicity of 0.10 μl VDC at 2, 4, and 8 hr compared to both the vehicle control and VA treatment alone (Fig. 2). The toxicity of 0.05 μl VDC was significantly increased at 8 hr compared to VA alone, while VA did not cause a significant K+ leakage in slices exposed to 0.025 μl VDC.

Effects of GdCl3 and VA Pretreatment on VDC Toxicity in Liver Slices

GdCl3 + VA pretreatment alone did not affect the viability of liver slices (Fig. 3). At 4 hr, intracellular K+ concentration in slices exposed to VA + 0.1 μl VDC was decreased to 29% of the control value. With GdCl3 pretreatment, K+/DNA was elevated to 53% of the control. A similar reversal by GdCl3 of VA-potentiated toxicity of 0.05 μl VDC was observed at 4 hr (64% decrease after VA, which was elevated to 96% after GdCl3). At 8 hr, GdCl3 did not reverse the VA-potentiated toxicity of 0.1 μl VDC. However, at 8 hr, the increase in the leakage of K+ caused by VA + 0.05 μl VDC (46%) was reversed by GdCl3 (71%). Similarly, VA induced K+ leakage of 0.025 μl VDC was reversed from 75% to the control value at 8 hr.

Histological Analysis of Liver Slices

Grading of the centrilobular necrosis in liver slices after VDC is summarized in Table 2. Exposure to VDC resulted in centrilobular necrosis of the liver slices. In liver slices
from vehicle pretreated rats no lesions were observed up to 4 hr, while minimal necrotic lesions were observed at 8 hr with exposure to 0.025 μl or 0.05 μl VDC. In liver slices exposed to 0.1 μl VDC minimal lesions are observed at 4 hr, while the lesions progressed mainly to moderate necrosis at 8 hr.

VA alone did not cause any necrotic changes up to 8 hr (Fig. 4A), except fatty accumulations in some hepatocytes. Pretreatment with VA markedly increased the extent of the necrotic lesions after exposure to VDC. The extent of the centrilobular lesion increased with the quantity of VDC and the time of exposure. Exposure to 0.025 μl VDC resulted in a few necrotic cells near the central veins (minimal necrosis) and the appearance of vacuolation in the midzonal hepatocytes. In slices exposed to 0.05 μl VDC minimal necrotic changes were seen with increases in the vacuolar changes in the midzonal area (Fig. 4B). At 4 hr treatment with 0.025 μl VDC resulted in mild necrotic changes (Fig. 4C) which progressed to moderate necrosis at 8 hr. Liver slices exposed to 0.05 μl VDC, minimal changes were seen at 2 hr, while at 4 hr lesions increased to moderate (Fig. 4D) or marked. At 8 hr, with the above treatment all slices were affected severely (Fig. 4E). With 0.1 μl VDC exposure, liver slices had moderate lesions as early as 2 hr. At 4 and 8 hr all liver slices had severe necrotic lesions.

Effects of VA and GdCl₃ + VA Pretreatments on VDC Hepatotoxicity in Vivo

VA alone did not cause any hepatotoxicity (Fig. 5). In rats treated with 200 mg/kg VDC alone one animal had slightly elevated ALT (358 IU/liter). Treatment with 100 mg/kg VDC alone did not result in any toxicity. VA pretreatment greatly enhanced the hepatotoxicity of both 200 and 100 mg/kg VDC. Mortality in the group which received VA + 200 mg/kg VDC was 4/5 at 24 hr, and in the group which received VA + 100 mg/kg VDC 3 of 4 died after 24 hr.

Treatment with GdCl₃ alone did not result in an elevation of ALT (results not shown). In the group of animals that received GdCl₃ + VA + 200 mg/kg VDC, 2/4 rats died after 24 hr. The ALT levels of the animals which survived after the above treatment were decreased by only 14% compared to VA + 200 mg/kg VDC treatment. Pretreatment with GdCl₃ decreased the VA potentiated hepatotoxicity of VA + 100 mg/kg VDC. All animals which received GdCl₃ + VA + 100 mg/kg VDC survived. The ALT levels of these animals were decreased by 72% compared to VA + 100 mg/kg VDC-treated animals.

Since VA caused mortality in 100 and 200 mg/kg VDC-treated rats, the effects of VA or GdCl₃ + VA on 50 mg/kg VDC was studied. Treatment with 50 mg/kg VDC had no effect on ALT. Pretreatment with VA significantly elevated ALT compared to vehicle + 50 mg/kg VDC. GdCl₃ pretreatment almost completely reversed the VA-induced hepatotoxicity in 50 mg/kg VDC-treated rats. The ALT levels of GdCl₃ + VA + 50 mg/kg VDC-treated rats were dropped to 93% lower than the value of VA + 50 mg/kg VDC treatment.

**Histological Analysis of the Liver**

VA pretreatment resulted in centrilobular necrosis of the livers of VDC treated rats at 24 hr (Table 3). In rats which received VA only (Fig. 6B) the livers did not differ much from those of vehicle controls (Fig. 6A), except that the former livers had some fatty accumulations in hepatocytes. Rats which received 50 or 100 mg/kg VDC had only minimal changes, which comprised of a few necrotic cells and an infiltration of a few inflammatory cells which consisted mainly of neutrophils around the central veins (Fig. 6C). In the livers of rats which received 200 mg/kg VDC mild necrotic changes were observed which consisted of a few layers of necrotic cells around central veins and inflammatory cells (Fig. 6D).

**TABLE 3**

<table>
<thead>
<tr>
<th>VDC (mg/kg):</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment:</td>
<td>C</td>
<td>VA</td>
<td>GdCl₃</td>
<td>C</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0 (normal)</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
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<tr>
<td>+1 (minimal)</td>
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<tr>
<td>+2 (mild)</td>
<td></td>
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<tr>
<td>+3 (moderate)</td>
<td></td>
<td></td>
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<tr>
<td>+4 (marked)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+5 (severe)</td>
<td></td>
<td></td>
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</tbody>
</table>

*Note. Rats were pretreated with vehicle (C), VA (250,000 IU/kg), or GdCl₃ (10 mg/kg) + VA 24 hr before VDC. Rats were euthanized 24 hr after VDC and the livers were fixed in 10% Formalin, processed for light microscopy, and stained with H&E.*

**Effects of VA and GdCl₃ + VA Pretreatments on VDC Hepatotoxicity in Vivo**

VA alone did not cause any necrotic changes up to 8 hr (Fig. 4A), except fatty accumulations in some hepatocytes. Pretreatment with VA markedly increased the extent of the necrotic lesions after exposure to VDC. The extent of the centrilobular lesion increased with the quantity of VDC and the time of exposure. Exposure to 0.025 μl VDC resulted in a few necrotic cells near the central veins (minimal necrosis) and the appearance of vacuolation in the midzonal hepatocytes. In slices exposed to 0.05 μl VDC minimal necrotic changes were seen with increases in the vacuolar changes in the midzonal area (Fig. 4B). At 4 hr treatment with 0.025 μl VDC resulted in mild necrotic changes (Fig. 4C) which progressed to moderate necrosis at 8 hr. Liver slices exposed to 0.05 μl VDC, minimal changes were seen at 2 hr, while at 4 hr lesions increased to moderate (Fig. 4D) or marked. At 8 hr, with the above treatment all slices were affected severely (Fig. 4E). With 0.1 μl VDC exposure, liver slices had moderate lesions as early as 2 hr. At 4 and 8 hr all liver slices had severe necrotic lesions.

**Histological Analysis of the Liver**

VA pretreatment resulted in centrilobular necrosis of the livers of VDC treated rats at 24 hr (Table 3). In rats which received VA only (Fig. 6B) the livers did not differ much from those of vehicle controls (Fig. 6A), except that the former livers had some fatty accumulations in hepatocytes. Rats which received 50 or 100 mg/kg VDC had only minimal changes, which comprised of a few necrotic cells and an infiltration of a few inflammatory cells which consisted mainly of neutrophils around the central veins (Fig. 6C). In the livers of rats which received 200 mg/kg VDC mild necrotic changes were observed which consisted of a few layers of necrotic cells around central veins and inflammatory cells (Fig. 6D).
VA pretreatment increased the extent of necrotic lesions depending on the dose of VDC. In VA + 50 mg/kg treated rats mild to moderate necrotic lesions are observed (Fig. 7A) while the rats which survived VA + 100 mg/kg VDC or VA + 200 mg/kg VDC exhibited marked (Fig. 7B) and severe (Fig. 7C) centrilobular lesions, respectively. While GdCl₃ alone did not cause any pathological lesions in the liver (results not shown), pretreatment with it resulted in the reduction of the extent of the lesion in VA + VDC-treated rats. In rats which received GdCl₃ + VA + 50 mg/kg VDC, minimal necrotic lesions were observed (Fig. 7D). In two of the rats which survived after GdCl₃ + VA + 100 mg/kg, the lesion severity decreased to moderate (Fig. 7E). In two rats which survived after GdCl₃ + VA + 200 mg/kg VDC lesion severity decreased to marked necrosis (Fig. 7F).

**PNP Hydroxylation by Liver Slices**

Both VA and pyridine pretreatment (Kim and Novak, 1990), which result in the specific induction of cytochrome P450IIE1, resulted in a twofold increase in PNP hydroxylation compared to control (Fig. 8) indicating that p-nitrophenol as well as VA increased the catalytic activity specific to cytochrome P450IIE1.

**Western Blot Analysis of Cytochrome P450IIE1 in Liver Slices**

Western blot analysis of cytochrome P450IIE1 protein indicated protein bands from control and VA pretreated rats corresponding to the band from pyridine pretreated rat (Fig. 9). Densitometric analysis demonstrated that there is a 2.4-fold increase in the intensity of bands from VA-pretreated rats compared to the control rats. This correlated well with the increase in PNP hydroxylation seen in VA-pretreated liver slices.

**Effects of Inhibition of Cytochrome P450IIE1 on VA Potentiated VDC Hepatotoxicity in Vivo**

VA pretreatment resulted in a significant elevation of plasma ALT compared to vehicle treatment (Fig. 10). DAS significantly decreased ALT in rats which received VA +
FIG. 7. Histological changes in the liver after VA + VDC or GdCl₃ + VA + VDC. Rats were treated with VA (250,000 IU po) and VDC was given 24 hr after VA. Another group of rats received GdCl₃ (10 mg/kg iv) just before treatment with VA and VDC was given 24 hr later. (A) Liver after treatment with VA + 50 mg/kg VDC showing mild necrosis. (B) Liver after treatment with 100 mg/kg VDC showing marked necrosis. (C) Liver after treatment with 200 mg/kg VDC showing severe necrosis. (D) Liver after treatment with GdCl₃ + VA + 50 mg/kg VDC. Note that the lesion has decreased in extent (minimal necrosis) compared to A. (E) Liver after treatment with GdCl₃ + VA + 100 mg/kg VDC. Note that the lesion has decreased compared to B, to a moderate lesion. (F) Liver after treatment with GdCl₃ + VA + 200 mg/kg VDC. Note that the extent of the lesion has decreased compared to C, to a marked lesion. Arrow points to the edge of the lesion. Original magnification, ×100.

VDC compared to rats which received CO + VA + VDC. This indicated that when VA induced cytochrome P450IIE1 activity is inhibited by DAS, there was reversal of the VA-potentiated VDC hepatotoxicity.

DISCUSSION

VA alone did not cause any liver damage, either in vivo or in vitro. A single treatment of VA resulted in a significant increase in VDC hepatotoxicity, both in vivo and in vitro. At 8 hr, VDC alone resulted in toxic injury to liver slices, except the two lowest concentrations as indicated by increased loss of intracellular K⁺. VA pretreatment significantly enhanced the toxicity of lower concentrations of VDC in liver slices at earlier times. A similar increase in VDC hepatotoxicity is seen in the in vivo study. There was a massive increase in toxicity in the group of rats which received VA and the higher doses of VDC with an increase in mortality and a dramatic increase in plasma ALT in the rats which survived. There was no mortality in the group which received VA and the lower dose of VDC, although ALT levels were significantly elevated compared to the control.

VDC treatment results in centrilobular necrosis of the liver (Reynolds et al., 1975). Similar necrotic lesions were observed after VA pretreatment and exposure to VDC, both in vivo and in liver slices. The extent of the centrilobular lesions depended on the amount of VDC in the in vivo study, and on the quantity and the time of exposure to VDC in the in vitro study. Although VA + 0.025 μl VDC did not cause a significant K⁺ leakage, mild to moderate necrotic lesions
were observed in liver slices at 4 and 8 hr. This may be due to the fact that a comparatively smaller number of hepatocytes were damaged and the overall K⁺ levels were not affected much. The necrotic lesions appear to be more severe in liver slices from VA-pretreated rats exposed to 0.05 and 0.1 μl VDC at 8 hr compared to the hepatic lesions observed in the in vivo study. This may be due to the fact that most of the VDC is exhaled unchanged in vivo. After ip administration of a 350 mg/kg dose of VDC, about 90% was exhaled as unchanged VDC (Jones and Hathway, 1978), and the delivery of VDC to the liver may be limited. In our sealed incubation system VDC concentrations may not change much, especially the higher concentrations of VDC, thus causing more damage to liver slices. In rats which are treated with hepatotoxic chemicals such as CCl₄ (Edwards et al., 1993) or VDC (Wijeweera, 1992), there is recruitment of other macrophages and neutrophils into the liver which exacerbate the injury. However, in liver slices, the injury proceeded without additional inflammatory infiltrations into the tissue. However, Kupffer cells already present in VA-pretreated liver slices may have contributed to hepatocellular damage.

There may be several mechanisms responsible for the observed potentiation of VDC hepatotoxicity after VA pretreatment. VA is known to activate the Kupffer cells (Earnest et al., 1986), the resident macrophages of the liver, and they may have had a role in the observed liver injury in the present study. Activated Kupffer cells can produce reactive oxygen species (Mobley et al., 1991), cytokines (Laskin, 1990), and proteolytic enzymes (Tanner et al., 1981) which can cause damage to the hepatocytes. Immunohistochemical staining showed a significant increase in the number of Kupffer cells in the centrilobular region of the liver of rats given a single treatment of VA and CCl₄ compared to rats which received CCl₄ alone, while GdCl₃ resulted in a drastic reduction in the number of Kupffer cells (Badger et al., 1996).

Treatment of rats with polyethylene glycol-coupled superoxide dismutase or catalase has been shown to block CCl₄-induced liver injury in VA-pretreated rats (ElSisi et al., 1993c). Inactivation of Kupffer cells by treatment with GdCl₃ (Edwards et al., 1993) or methyl palmitate (ElSisi et al., 1993c) reversed VA-potentiated CCl₄ hepatotoxicity. In the present study, GdCl₃ pretreatment did not completely reverse VA-potentiated VDC hepatotoxicity either in vitro or in vivo. In the in vivo experiments, increase in plasma ALT was partially blocked and the centrilobular lesions were only decreased in size. In the in vitro experiments GdCl₃ resulted in a partial reversal of K⁺ leakage from liver slices after VA and VDC. Thus, while Kupffer cells may play a role in potentiation, other mechanisms also may be responsible for the enhancement of VDC hepatotoxicity after VA pretreatment.

It is possible that a single treatment of VA may cause changes in the liver that alter bioactivation or the detoxification pathways of VDC. Conjugation with GSH is the major detoxification pathway for reactive VDC metabolites (Jones and Hathway, 1978). Previous studies have shown that VA does not change the concentrations of hepatoprotective agents in the liver such as vitamin E or GSH (ElSisi et al., 1993b). Therefore, the increase in toxicity of VDC after VA

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**FIG. 9.** Western blot analysis of cytochrome P450IIE1 from liver slices from rats treated with VA or pyridine. Control rats received the vehicle. Liver slices were homogenized and centrifuged at 10,000g, and 40 μg of proteins from the supernatant was loaded onto each lane of the gel and subjected to SDS-PAGE. Cytochrome P450IIE1 bands were visualized by immunoperoxidase staining method. Lane a, molecular weight markers; lane b, sample from a pyridine-treated rat showing the position of the cytochrome P450IIE1 protein band; lanes c–e, samples from three control rats; lanes f–h, samples from three VA-treated rats. Positions of 66- and 45-kDa molecular weight markers are shown.

**FIG. 10.** Effects of DAS treatment on VA-potentiated VDC hepatotoxicity in vivo. Rats were pretreated with VA 24 hr before the administration of 50 mg/kg VDC. Four hours before VDC, rats were treated with 100 mg/kg DAS in 1 ml corn oil (CO) ip. Control rats received the vehicle treatments. Twenty-four hours after VDC rats were euthanized by an overdose of pentobarbital, and plasma ALT was measured. * indicates significantly different from vehicle + CO + VDC. ** indicates significantly different from VA + CO + VDC.
pretreatment appears not to result from a reduction in hepatic GSH. Similarly, because GdCl₃ does not increase liver GSH levels (Edwards et al., 1993), partial protection seen after GdCl₃ cannot be due to an increase in the detoxification of VDC metabolites.

Since Badger et al. (1995, 1996; Badger and Sipes, 1996) reported that a single treatment of rats with VA increased the concentration and activity of hepatic cytochrome P450IIIE1, we investigated whether such effects could be observed in liver slices. VA pretreatment increased both the cytochrome P450IIIE1 protein content and its catalytic activity as studied by PNP hydroxylation in liver slices. Treatment with other chemicals such as pyridine for 1 day is known to induce cytochrome P450IIIE1 contents in rat liver (Kim and Novak, 1990). Other studies have shown that when rats were given a diet high in VA, they had increased levels of total cytochrome P450 (Leo et al., 1984). VA treatment also induces P4502C7 isozyme in primary rat hepatocytes (Westin et al., 1993) and P450III A2 in rat liver (Murray et al., 1991). Induction of cytochrome P450IIIE1 by VA pretreatment can result in an increased bioactivation of VDC, resulting in an increase in the amount of toxic metabolites that cause damage to hepatocytes. Cytochrome P450 is localized in the centrilobular region of the liver (Baron et al., 1978). This correlates well with our observation that the necrotic lesion is localized to this area of the liver in VA-pretreated rats or liver slices exposed to VDC. Inhibition of cytochrome P450IIIE1 with DAS resulted in a complete reversal of VA potentiation of VDC hepatotoxicity, indicating that induction of cytochrome P450IIIE1 by VA plays a role in the observed VA potentiation of VDC hepatotoxicity.

In summary, more than one mechanism may be responsible for the enhancement of VDC hepatotoxicity after VA pretreatment. It is clear that even a single treatment with VA can cause an increase in the toxicity of VDC, and this may be true for other toxicants as well. This may have important consequences especially in people who consume large daily doses of over-the-counter VA supplements because there is possible exposure to environmental contaminants and other drugs which may interact to cause liver damage.

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