Role of Cytochrome P4502E1 in Retinol’s Attenuation of Carbon Tetrachloride-Induced Hepatotoxicity in the Swiss Webster Mouse

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In the mouse, retinol administration attenuates carbon tetrachloride (CCl4)-induced hepatic injury. We have investigated the role of cytochrome P4502E1 (CYP2E1) in this interaction. Male Swiss Webster mice were administered retinol (75 mg/kg/d) or vehicle for 3 days prior to CCl4 (30 μl/kg, ip). Hepatotoxicity produced by CCl4 was assessed by plasma alanine aminotransferase (ALT) activity and light microscopy (ALT activity of 1391 ± 430 vs. 274 ± 92 IU/L for vehicle + CCl4 and retinol + CCl4 treatments respectively, p < 0.05). Retinol’s attenuation of liver injury was maintained when CCl4 was administered 48 h after the conclusion of the retinol pretreatment. Aniline hydroxylation activity, an indicator of CYP2E1 catalytic activity, determined on day 4 was 33.8% of untreated control in vehicle + CCl4, treatments while the retinol + CCl4 treatment group was 94.2% of untreated control. Additionally, CYP2E1 immunoreactive protein was 78% lower in vehicle + CCl4, vs. retinol + CCl4, treatment groups. Attenuation of potentiated hepatotoxicity was also observed when CYP2E1 was induced by acetone (ALT activity of 3119 ± 1066 vs. 247 ± 77 IU/L for vehicle and retinol treatments respectively, p < 0.05). In the mouse, retinol itself does not alter constitutive or inducible CYP2E1 expression. However, in combination with CCl4, retinol reduces the amount of CCl4, bioactivated to its toxic metabolite. We conclude that retinol attenuates CCl4-induced hepatotoxicity by causing a decrease in CCl4 bioactivation but does not cause a decrease in CYP2E1 expression.

Key Words: carbon tetrachloride; cytochrome P4502E1; retinol; Swiss Webster mouse; attenuation; hepatotoxicity.

Retinol (Vitamin A) is a naturally occurring, fat-soluble vitamin contained in many different vegetables. It can also be purchased over-the-counter as a dietary supplement. Adequate retinol consumption is required for the maintenance of normal growth, reproduction, cellular differentiation, and immuno-competence (Lotan, 1980). Pharmacological doses of vitamin A and other retinoids have been used clinically for their therapeutic activity in the treatment of dermatological disorders and as adjuvants in cancer chemotherapy (Bollag, 1983; Moon et al., 1997; Newton and Sporn, 1979). Additionally, individuals commonly use dietary supplementation of retinol for either self-treatment or for the prevention of non-specific illness such as the common cold. Therefore, individuals may ingest large quantities of retinol for a variety of reasons. Once in the body, the liver is the principle organ responsible for the storage of retinol. During hypervitaminosis A, the liver’s capacity to store retinyl palmitate is exceeded, which can then lead to hepatotoxicity (Russell et al., 1974). However, clinical data on the risk of developing significant liver disease from chronic ingestion of excess amounts of retinol has shown conflicting results (Krause, 1965; Minuk et al., 1988). Therefore, the risk of hepatotoxicity associated with excess consumption of retinol supplements remains uncertain.

Numerous studies in a variety of rodent species have demonstrated that retinol pretreatment modulates the hepatotoxicity of many different drugs and chemicals. For example, retinol potentiated the liver injury induced by ethanol (Leo and Lieber, 1983), carbon tetrachloride (CCl4) (Sipes et al., 1990), and acetaminophen (El Sisi et al., 1993a) in the Sprague-Dawley rat. Serum enzymes and liver morphology demonstrated that subacute hypervitaminosis A was not producing the liver injury. Further studies by Badger et al. (1996) demonstrated that 1 day of retinol pretreatment also potentiated CCl4-induced hepatotoxicity. This study demonstrated that CYP2E1 polypeptide levels were increased in the rat following retinol exposure. The authors concluded that this induction of CYP2E1 expression by retinol played an important role in retinol’s potentiation of CCl4-induced hepatotoxicity. Retinol pretreatment has the opposite effect on CCl4-induced hepatotoxicity in mice. Experiments in various strains of mice have shown that retinol attenuated CCl4-induced hepatotoxicity (Hooser et al., 1994), indicating that the ability of retinol to modulate CCl4-induced hepatotoxicity was species-specific. Further examination of this interaction in the male Swiss Webster mouse indicated that 3 days of retinol (75 mg/kg/day) pretreatment was the minimum dose required to attenuate CCl4-induced hepatotoxicity (Rosengren et al., 1995).

The present study was designed to investigate the mecha-
nism responsible for retinol’s attenuation of CCl4-induced liver injury in the Swiss Webster mouse. CCl4, an industrial solvent, utilizes CYP2E1 to undergo reductive dechlorination, which produces reactive intermediates that cause hepatic and renal damage (Ekstrom et al., 1989; Guengerich et al., 1991; Recknagel and Glende, 1973; Sipes et al., 1977). Liver injury induced by CCl4 is manifested as both centrilobular necrosis and the accumulation of fat, and bioactivation is an essential prerequisite to the hepatotoxicity of CCl4, both in vivo and in vitro (Manno et al., 1988).

Numerous studies with retinoids have shown that retinol and its metabolites react in a species-specific manner with cytochrome P450 isozymes. For example, in rats, retinyl acetate (25 IU/g) increased the microsomal expression of CYP3A2 (Murray et al., 1991). In addition, rat CYP2B1 and CYP2C7, and human CYP2C8 have all demonstrated an ability to metabolize retinol and retinoic acid to more polar products (Leo et al., 1984, 1989; Leo and Lieber, 1985). In the rabbit, however, CYP1A2 and CYP3A6 catalyze the conversion of retinol to retinoic acid (Roberts et al., 1991b). Additionally, in Syrian hamsters retinyl palmitate markedly increased CYP2A1 polypeptide levels (Ushio et al., 1996). More recently, CYP26 has been identified in mouse and human liver and, in the adult liver, its transcriptional expression is induced by retinoic acid (Haque et al., 1998; Ray et al., 1997). These studies provide insight into the interactions between retinoids and various CYP450 isozymes in different species.

Extensive investigations with either retinoids and/or CCl4 have primarily used the Sprague-Dawley rat as the animal model. For example, the administration of a small dose of CCl4 results in a biphasic stimulation of hepatic cell division, which leads to tissue repair and recovery from tissue injury (Mehendale, 1994). Further observations led to the proposal that the critical mechanism in this autoprotection model was not decreased liver injury associated with the administration of a large lethal dose, but instead was linked to a more rapid recovery from equivalent liver injury (Mehendale et al., 1994). The specific mechanism of retinol’s potentiation of CCl4-induced hepatotoxicity in the rat has been well characterized and involves both Kupffer-cell activation and CYP2E1 induction (Badger et al., 1997, 1996; El Sisi et al., 1993a,b; Hooser et al., 1994; Wijeweera et al., 1996). Since rats and mice have opposite responses to the combination of retinol and CCl4, it cannot be assumed that retinol will have an identical effect on the CYP450 system in the mouse when compared to responses reported in the rat. Moreover, since various CYP450 isozymes are involved in the metabolism of retinoids in various species, it is logical to examine retinol’s effect on CYP2E1 in the mouse. Therefore, we aim to determine the mechanism by which retinol attenuates CCl4-induced hepatotoxicity by focusing on CYP2E1, the major enzyme responsible for the bioactivation of CCl4.

**MATERIALS AND METHODS**

**Chemicals.** HPLC grade carbon tetrachloride was obtained from Merck (Darmstadt, Germany). Acetone was purchased from BDH Laboratory Supplies (Poole, England). The ALT diagnostic kit (procedure No. 59-UV), polyethylene glycol, Tris, gadolinium chloride, aniline-HCl, phenol, and rabbit anti-goat IgG (alkaline phosphatase conjugate) were purchased from Sigma Chemicals (St. Louis, MO). Nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), sodium dodecylsulfate (SDS), β-mercaptoethanol, glycine, acrylamide/bis, ammonium persulfate, TEMED, molecular weight standards, and avidin-alkaline phosphatase conjugate were purchased from Bio Rad (Hercules, CA). Retinol was provided as Aquasol A drops and was purchased from Astra USA, Inc. (Westborough, MA). p-Aminophenol was purchased by Aldrich Chemical Co. (Milwaukee, WI). Anti-rat CYP2E1 primary antibody was purchased from Gentest (Woburn, MA).

**Animals.** Male Swiss Webster mice (5–6 weeks old, 25 g) were obtained from the Department of Laboratory Animal Sciences (Otago University, Dunedin). They were housed, in approved animal facility, in polycarbonate cages with hardwood bedding and maintained at 21–24°C under a 12-h light/dark cycle. These animals received ad libitum food and water and were maintained on a 12-h light/dark cycle. Briefly, immediately following blood collection, mouse livers were perfused with 1.15% KCl and then removed, trimmed of debris, blotted, weighed and immediately stored on ice in 1.15% KCl. The livers were then rinsed in fresh 1.15% KCl and minced with scissors. The KCl was replaced with 2 ml of TRIS (0.1 M Tris–HCl, 0.1 M KCl, 1 mM EDTA, 20 μM BHT, 1 mM dithiothreitol, pH 7.4) buffer per gram of liver tissue. Livers were then homogenized using a teflon-glass homogenizer and the homogenates were subjected to differential centrifugation. Protein concentration of the resulting microsomal pellet was determined using the bicinchoninic (BCA) protein assay (Smith et al., 1985) and the samples were stored at –80°C until use.

**Microsomal enzyme assays.** Since CYP2E1 catalyses the conversion of aniline to p-aminophenol, aniline hydroxylation was used to determine the catalytic activity of CYP2E1. The assay was conducted as described (Schenkman et al., 1967). All reagents were prepared fresh and all light-sensitive
solutions were kept in the dark until used. Reaction mixtures, in a total volume of 1 ml, contained 0.5-mg microsomal protein, 200-mM aniline-HCl, 50 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH (samples only), which were then vortexed and incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.3 ml of cold 20% trichloroacetic acid (TCA). After centrifugation at 3000 rpm for 10 min, 1 ml of the supernatant was added to 100 µl phenol (2% phenol in 10 N NaOH) followed by the addition of 200 µl of 2.5 M Na2CO3.

The tubes were vortexed and the color was allowed to develop for exactly 30 min. The formation of p-aminophenol was determined on a Spectromax Plus spectrophotometer at a wavelength of 630 nm. The product of the reaction, p-aminophenol, was used for the preparation of the standard curve. Results are expressed as nmol/mg protein/min.

Electrophoresis and Western blotting. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as previously described (Laemmli, 1970). Briefly, 10 µg of microsomal protein was loaded into wells of 10% polyacrylamide gels. The gels were run at 200V until the dye front reached the bottom of the gel. CYP2E1 polypeptide levels present in the hepatic microsomes were quantified by Western immunoblotting. This method was conducted as described (Towbin et al., 1979). Briefly, the transfer of proteins from the gel to nitrocellulose was carried out using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. Blots were then blocked with 5% nonfat milk for 1 h. Membranes were then incubated in rabbit-anti-rat CYP2E1 primary antibody (Gentest), diluted 1:500 in blocking solution (0.01% milk powder in TBS) for one h with shaking. The nitrocellulose was then washed 3×, 5-min each time, with TTBS (0.1% Tween 20, 0.55 mM Tris, 200 mM NaCl, pH 7.4), at room temperature. The nitrocellulose was then incubated for 1 h with secondary antibody (anti-goat IgG, an alkaline phosphatase conjugate). After washing, alkaline phosphatase color-development reagents (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt, and p-nitro blue tetrazolium chloride) were added and the intensity of the bands was analyzed by densitometry.

Statistical analysis. Individual groups were analyzed using a 2-way ANOVA coupled with the Student-Newman-Keuls post-hoc test and p < 0.05 was the minimum requirement for a statistically significant difference.

RESULTS

Previous experiments have shown that 3 days of retinol (75 mg/kg) pretreatment was the minimum dose required to attenuate CCl4-induced hepatotoxicity as determined by plasma ALT activity and the histopathology of liver sections (Ronggren et al., 1995). To determine the duration of retinol’s protective effect, mice were dosed with retinol (75 mg/kg/d, 3 d) and were then given a CCl4 (30 µl/kg) challenge 24, 48, and 72 h after the last dose of retinol. Attenuation of CCl4-induced hepatotoxicity was maintained when CCl4 was given 24 h after the last dose of retinol (ALT activities of 1654 ± 532 vs. 376 ± 91 IU/L for vehicle + CCl4 vs. retinol + CCl4 respectively) (Fig. 1). However, retinol lost its ability to attenuate CCl4-induced hepatotoxicity when CCl4 was administered 72 h after the last dose of retinol (ALT activities of 317 ± 127 vs. 1480 ± 376 IU/L for vehicle + CCl4 vs. retinol + CCl4 respectively). Histological evaluation of liver sections from mice treated with CCl4 48 h after the vehicle of retinol showed severe centrilobular necrosis, while CCl4 administered 48 h after retinol pretreatment showed mild necrosis (Fig. 2). However, both groups showed severe centrilobular necrosis when retinol was administered 72 h after the oral dose of retinol (Fig. 2). To determine if retinol’s attenuation of CCl4-induced hepatotoxicity only occurred when there was a significant induction of hepatotoxicity, mice were dosed with a lower dose of CCl4 (10 µl/kg), which produced very minimal hepatic damage. Under these conditions, retinol significantly decreased this minimal hepatotoxicity (ALT activities of 347 ± 77 and 31 ± 7 IU/L for vehicle + CCl4 vs. retinol + CCl4 respectively) (Fig. 3).

Investigations next focused on the ability of retinol to attenuate CCl4-induced hepatotoxicity which was potentiated. When CCl4 hepatotoxicity was potentiated by the classical P450 inducer phenobarbital (80 mg/kg/d, 3d, ip), retinol continued to attenuate the resultant hepatotoxicity (Fig. 4). Additionally, when the hepatotoxicity was potentiated by induction of CYP2E1 with a single oral dose of acetone (4.8 g/kg), retinol maintained its ability to protect Swiss Webster mice from the elevated liver injury (ALT activities of 3119 ± 1067 and 247 ± 77 IU/L for vehicle + CCl4 vs. retinol + acetone + CCl4 respectively) (Fig. 5). Studies to determine the mechanism of retinol’s attenuation of CCl4-induced liver injury primarily focused on the role of CYP2E1. Therefore, further experiments investigated the changes in the catalytic activity and polypeptide levels of CYP2E1 in individual mice from all treatment groups. Aniline hydroxylation activity, an indicator of CYP2E1 catalytic activity, determined on day 4 was 33.8% of untreated control in vehicle + CCl4 treatments while the retinol + CCl4 treatment group was 94.2% of untreated control (Table 1). Furthermore, aniline hydroxylation

FIG. 1. Retinol’s attenuation of CCl4-induced hepatotoxicity as assessed by plasma ALT activity. Mice were gavaged with retinol (75 mg/kg/d) for 3 days. Twenty-four, 48 or 72 h after the last oral dosage, CCl4 (30 µl/kg, ip) was administered. Necropsies were performed 24 h after the administration of CCl4. The bars represent the mean ± SEM from 10 mice per group. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test. *Significantly different (p < 0.05) from the respective vehicle + CCl4-treated groups.
activity in microsomes from mice treated with retinol + CCl₄ was not different from that in mice treated with retinol only or from vehicle control groups (Table 1). Immunoblotting was performed to detect changes in the polypeptide levels of CYP2E1 in all treatment groups and the results demonstrated a similar trend. Scanning densitometry of the western blot (Fig. 6) showed that the polypeptide levels for CYP2E1 were reduced by 78% in the vehicle + CCl₄ group compared to the retinol + CCl₄ and control groups. This trend was supported by aniline hydroxylation activity determined in acetone-induced microsomes. Catalytic activity for CYP2E1 was 51.2% of acetone control in the vehicle + acetone + CCl₄ treatment group, while retinol + acetone + CCl₄ and other control groups remained unchanged from acetone control (Table 2). Enzymatic data obtained from microsomes prepared from animals which were administered CCl₄ 48 h after the last dose of retinol showed similar results. In these studies, vehicle + CCl₄ treatments reduced CYP2E1 polypeptide levels (Fig. 7) and decreased CYP2E1 catalytic activity (Table 3) compared to untreated control, while in retinol + CCl₄ treated mice these parameters remained not different from control. However, when CCl₄ was administered 72 h after the last dose of retinol both the retinol + CCl₄ and the vehicle + CCl₄ treatment groups showed a reduction in both catalytic activity (46–47%) (Table 3) and polypeptide levels (63–72%) (Fig. 8) for CYP2E1 when compared to untreated control.

**DISCUSSION**

Previous results from our laboratory have shown that in the mouse, retinol pretreatment modulates the hepatotoxicity induced by a variety of different chemicals (Rosengren et al., 1995). These findings demonstrated that retinol potentiated the hepatotoxicity of galactosamine, paracetamol and allyl alcohol,
while it attenuated the hepatotoxicity of CCl₄ and phalloidin. This work and other similar studies in the rat showed that the interaction between retinol and CCl₄ was species-specific. While retinol (75 mg/kg/d, 7 days) potentiated CCl₄-induced liver injury in 3 different strains of rat, it attenuated CCl₄-induced liver injury in 4 different strains of mice (Hooser et al., 1994). This correlates with the concept that the susceptibility to CCl₄-induced liver injury is dependent on the species (Zimmerman, 1978). For example, the mouse is much more sensitive to CCl₄-induced liver damage than the rat. Minimal hepatotoxicity is produced in the mouse after a 10–20 μl/kg dose (Roberts et al., 1991a; Shertzer et al., 1987), whilst in the rat, minimal hepatotoxicity is observed at doses of 100–200 μl/kg (Hooser et al., 1994; Soni and Mehendale, 1994; Thakore and Mehendale, 1991).

Experiments to determine the mechanism of retinol’s potentiation of CCl₄-induced hepatic injury in the rat showed that 1 day of retinol pretreatment increased both the initiation (bioactivation via induction of CYP2E1) and the progression (inflammatory-mediated increases in hepatocyte damage) of liver injury produced by CCl₄ (Badger et al., 1996). The authors also demonstrated that the degree of potentiation varied with the duration of retinol pretreatment. The results of this study demonstrate that in the rat, retinol alters both the initiation and progression of CCl₄-induced liver injury.

We were interested in determining why a similar dose of retinol-attenuated, CCl₄-induced hepatotoxicity in the mouse. Previously, we have shown that 3 days of retinol (75 mg/kg/d) was the minimum dose necessary to attenuate the hepatotoxicity of CCl₄ (Rosengren et al., 1995). It would be expected that the interaction between retinol and CCl₄ in the mouse would have an action distinct from that demonstrated in the rat. Therefore, our first aim was to further characterize this model of attenuated hepatotoxicity. The first aspect of this was to examine the duration of retinol’s protective effect. In these studies, retinol maintained its ability to attenuate CCl₄-induced hepatotoxicity when CCl₄ was administered up to 48 h after the conclusion of the retinol treatment. At 72 h following the retinol pretreatment, the protective effect was lost. These results further defined the attenuation model that now comprises 3 days of retinol pretreatment at 75 mg/kg/d, followed by CCl₄ administration within 48 h. This model was then used to focus on exactly how retinol attenuates CCl₄-induced liver injury.

To confirm that retinol’s attenuation of CCl₄-induced hepatotoxicity was not limited to situations where there was a significant induction of hepatotoxicity, mice were administered a lower dose of CCl₄ (10 μl/kg), which resulted in a very minimal level of initial hepatic injury. Under these conditions, retinol pretreatment continued to provide protection against minimal hepatic injury. This demonstrates that the effect observed in the rat (i.e., low level of hepatic injury potentiated by retinol) (Badger et al., 1996, 1997; El Sisi et al., 1993a,b; Hooser et al., 1994; Sipes et al., 1990) is not duplicated in the mouse. Instead, retinol consistently attenuates CCl₄-induced liver injury and this protection is not lesion-dependent since

![FIG. 3. Protective effect of retinol on mild CCl₄ hepatotoxicity as assessed by plasma ALT activity. Mice were gavaged with retinol (75 mg/kg/d) for 3 days. Twenty-four h after the last oral dosage, CCl₄ (10 μl/kg, ip) was administered. Necropsies were performed 24 h after the administration of CCl₄. The bars represent the mean ± SEM from 10 mice in each treatment group and 5 mice in each control group. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post hoc test. *Significantly different (p < 0.001) from the vehicle + CCl₄ treatment group.](image-url)
Protection from liver injury is always observed regardless of the severity of the initial injury. This adds further confirmation that the effect exhibited by the combination of retinol and CCl₄ in the mouse is not a function of dose and indeed is purely species-specific.

Since it was established that retinol attenuated low and moderate levels of hepatic injury, our next point of interest was to examine retinol’s ability to attenuate liver injury when the initial CCl₄-induced liver injury was potentiated by acetone. The results from these studies demonstrated that retinol was able to significantly diminish this increased liver injury. A similar effect was observed when CCl₄-induced hepatic injury was potentiated by pretreatment with phenobarbital. These studies indicated that under conditions of CYP2E1 induction and therefore an increase in the formation of the CCl₄ toxic metabolite, retinol maintains an ability to attenuate CCl₄-induced hepatic injury.

Since retinol continued to provide protection from CCl₄-induced hepatotoxicity in CYP2E1-induced mice, we decided to concentrate on how CYP2E1 responds to retinol treatment with and without further administration of CCl₄. Several studies have reported an increase in CYP2E1 expression in the rat.

### FIG. 5
Protective effect of retinol on acetone-induced potentiation of CCl₄ hepatotoxicity as assessed by plasma ALT activity. Mice were gavaged with retinol (75 mg/kg/d) for 3 days. Sixteen h prior to the dose of CCl₄ (30 μl/kg, ip) acetone was administered (4.8 g/kg, po). Mice dosed with corn oil, and acetone + corn oil served as controls. Necropsies were performed 24 h after the administration of CCl₄. The bars represent the mean ± SEM from 10 mice per treatment group and 5 mice per control group. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test. *Significantly decreased (p < 0.05) from respective vehicle + CCl₄-treated groups. **Significantly increased (p < 0.05) from the vehicle + CCl₄ treatment group.

### TABLE 1
Aniline Hydroxylation Activity following Retinol and CCl₄ Treatments

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>% of untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>Retinol + corn oil</td>
<td>14</td>
<td>95.4 ± 1.2</td>
</tr>
<tr>
<td>Vehicle + corn oil</td>
<td>14</td>
<td>96.8 ± 1.4</td>
</tr>
<tr>
<td>Retinol + CCl₄</td>
<td>14</td>
<td>94.2 ± 1.5</td>
</tr>
<tr>
<td>Vehicle + CCl₄</td>
<td>14</td>
<td>33.8 ± 5.3*</td>
</tr>
<tr>
<td>Acetone</td>
<td>8</td>
<td>367.5 ± 14.2**</td>
</tr>
</tbody>
</table>

*Note.* Values are the mean ± SEM. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test.

*Significantly decreased from other treatment groups, p < 0.05.

**Significantly increased from other treatment groups, p < 0.01.

Untreated control aniline hydroxylation activity was 2.03 ± 0.18 nmol/mg/min.

### FIG. 6
Western blot analysis of CYP2E1 in hepatic microsomes prepared from treated mice. Swiss Webster mice were gavaged with retinol (75 mg/kg/d) or vehicle for 3 days. On day 4, they were treated with CCl₄ (30 μl/kg, ip). Twenty-four h later, microsomes were prepared as described under Materials and Methods. Microsomal protein (10 μg) was loaded onto each lane of a 10% polyacrylamide gel and immunoblotted with anti-rat CYP2E1 primary antibody (Genestest). Protein was visualized with alkaline phosphatase color reagent and the bands were quantified by scanning densitometry. The positions of the molecular weight markers are indicated on the left (×10³ kDa). The lanes from left to right are retinol + CCl₄ (1–3) vehicle + CCl₄ (4–6), untreated (7), vehicle + corn oil (8) retinol + corn oil (9), and acetone (10). The arrow represents CYP2E1 and the bottom band is due to a minor cross reactivity with CYP2C11. The optical density of the bands is shown in the graph below the Western blot. The bars represent the mean ± SEM of the optical density for bands from similar treatment groups. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test. *Significantly decreased (p < 0.05) from all other treatment groups. **Significantly increased (p < 0.05) from all other treatment groups.
Aniline Hydroxylation Activity in CCl₄ Administration following Acetone Induction

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>% of acetone control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Retinol + acetone + corn oil</td>
<td>7</td>
<td>95.1 ± 5.7</td>
</tr>
<tr>
<td>Vehicle + acetone + corn oil</td>
<td>7</td>
<td>98.8 ± 2.5</td>
</tr>
<tr>
<td>Retinol + acetone + CCl₄</td>
<td>7</td>
<td>91.9 ± 1.8</td>
</tr>
<tr>
<td>Vehicle + acetone + CCl₄</td>
<td>7</td>
<td>51.2 ± 2.2*</td>
</tr>
</tbody>
</table>

Note. Values are the mean ± SEM. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test.
* Significantly decreased from other treatment groups, p < 0.05. Acetone control aniline hydroxylation activity was 12.44 ± 0.89 nmol/mg/min.

TABLE 2

after 1 day of retinol treatment (Badger et al., 1996; Wijeweera et al., 1996), so it seemed logical to determine if retinol had an effect on CYP2E1 expression in the mouse. Other groups have also shown that the administration of various retinoids altered CYP450 levels in different species (Murray et al., 1991; Ushio et al., 1996). Therefore, the concept of retinol altering the expression of CYP450 was not unprecedented. In our investigations, CYP2E1 catalytic activity and polypeptide levels were determined in individual mice taken from all treatment groups in the 3-day attenuation model. The results from the catalytic activity and immunoreactive protein data are similar. They both demonstrate that CCl₄ destroys CYP2E1 catalytic activity and polypeptide levels while retinol itself does not suppress the constitutive expression of CYP2E1.

Recent investigations in cyp2e1⁻/⁻ mice by Wong et al. (1998) conclusively demonstrated that CYP2E1 activity is required for the initiation of CCl₄-induced hepatotoxicity. When these knock-out mice were administered CCl₄ (1 ml/kg), no hepatotoxicity was observed compared to significant liver injury in the wild-type controls (ALT activities of 20,000 IU/L). CYP2E1 catalytic activity, as determined by p-nitrophenol hydroxylation, was decreased compared to the wild-type (Wong et al., 1998). This study supports our observations that CYP2E1 must bioactivate CCl₄ for hepatotoxicity to be observed. Our data indicates that when retinol is administered to mice, it prevents CYP2E1 bioactivation, since there is no subsequent loss of CYP2E1 catalytic activity or polypeptide levels in mice treated with retinol + CCl₄.

Our data is supported by studies by Tierney et al. (1992), who demonstrated that CCl₄ administration in male mice caused a destruction of 75% of the immunochemically detectable CYP2E1 protein. When the authors substituted CCl₄ with 1-aminobenzotriazole (ABT), a complete loss of p-nitrophenol activity was observed, but they reported only a 12% loss in CYP2E1 polypeptide levels. The authors concluded that CCl₄ inactivates CYP2E1 by heme alkylation of the protein, while ABT inactivates CYP450 by N-alkylation of the heme (Tierney et al., 1992). Consequently, after CCl₄ treatment, apoprotein and holoprotein levels are rapidly decreased but ABT treatment does not lead to a complete loss of CYP2E1; instead of being destroyed a stable apoprotein is formed. Our data is similar: we saw a 78% loss of immunochemically detectable protein via Western blotting after mice were treated with vehicle + CCl₄.

Interestingly, results obtained from Western blotting and aniline hydroxylation demonstrated that the retinol + CCl₄-treatment group had no decrease in CYP2E1 polypeptide levels or catalytic activity when compared to untreated and vehicle control mice. Therefore when retinol is administered, it attenuates CCl₄-induced hepatic injury by preventing the CYP2E1-catalyzed bioactivation of CCl₄. The data is further supported by the fact that a similar effect is observed when CCl₄ is administrated 48 h after the conclusion of the retinol pretreatment. By 72 h, the protective effect of retinol is lost and both

FIG. 7. Western blot analysis of CYP2E1 in hepatic microsomes prepared from treated mice. Swiss Webster mice were gavaged with retinol (75 mg/kg/d) or vehicle for 3 days. On day 5, they were treated with CCl₄ (30 μl/kg, ip). Twenty-four h later, microsomes were prepared as described under Materials and Methods. Microsomal protein (10 μg) was loaded onto each lane of a 10% polyacrylamide gel and immunoblotted with anti-rat CYP2E1 primary antibody (Genentest). Protein was visualized with alkaline phosphatase color reagent and the bands were quantified by scanning densitometry. The positions of the molecular weight markers are indicated on the left (×10⁷ kDa). The lanes from left to right are vehicle + CCl₄ (1–3), retinol + CCl₄ (4–6), untreated (7), and acetone (8). The arrow represents CYP2E1 and the bottom band is due to a minor cross reactivity with CYP2C11. The bars on the graph represent the mean ± SEM of the optical density for bands from similar treatment groups. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test. **Significantly increased (p < 0.05) from all other treatment groups. **Significantly increased (p < 0.05) from all other treatment groups.
the retinol + CCl₄ and the vehicle + CCl₄ groups show similar hepatotoxicity (ALT activity and histopathology) and CYP2E1 expression (catalytic activity and polypeptide levels). Apparently, if CCl₄ is administered 72 h after the conclusion of the retinol pretreatment, retinol no longer has the ability to prevent the bioactivation of CCl₄. Previous results have shown that retinol levels in the mouse liver are significantly decreased at 4 days after the 3-day pretreatment when compared to the retinol levels at 24 h following the 3-day pretreatment (Rosengren et al., 1995). Conversely, the levels of retinyl palmitate (the storage form of retinol) were unchanged 4 days after the retinol pretreatment. This data implies that the protective effect of retinol may be linked to the hepatic levels of retinol and may not be associated with the levels of retinyl palmitate. The fact that the retinol levels and the return of CCl₄-induced hepatotoxicity occur at the same time point provide support for this theory.

These studies further show that retinol itself does not alter CYP2E1 expression in the mouse, since the values for both catalytic activity and immunoreactive protein in mice treated with only retinol were not different from control. Moreover, retinol’s effect on CYP2E1 is species-specific since there is no induction of CYP2E1 catalytic activity or polypeptide levels after 3 days of retinol pretreatment. In contrast, induction of CYP2E1 has been observed after 1 day of retinol treatment in the rat (Badger et al., 1996; Wijeweera et al., 1996), indicating that this system is one in which the two species react differently following exposure to retinol.

Other compounds, such as 2-(allylthio)pyrazine (2-AP), protect against CCl₄-induced hepatotoxicity in the mouse by suppressing constitutive and inducible CYP2E1 expression and by inducing microsomal epoxide hydrolase and glutathione s-transferase (Kim et al., 1997). In these studies, hepatic GSH was also elevated in 2-AP + CCl₄-treated mice compared to CCl₄-treated mice. Our studies with retinol also show protection against CCl₄-induced hepatotoxicity and CCl₄ bioactivation is decreased, even though retinol does not alter constitutive or inducible CYP2E1 expression. In addition, previous studies have shown that retinol itself does not alter GSH levels in the mouse (Rosengren and Sipes, 1995). Therefore, retinol is acting differently from 2-AP in its attenuation of CCl₄-induced hepatotoxicity.

Our data demonstrates that retinol’s primary role in its protection of CCl₄-induced liver injury is one of preventing the CYP2E1 bioactivation of CCl₄. If it were acting as an anti-oxidant and scavenging the trichloromethyl radical, then the catalytic activity and polypeptide levels of CYP2E1 would be decreased in the retinol + CCl₄ treatment group. Since these parameters are not decreased, then there is a decrease in the production of the toxic metabolite and not a decrease in the hepatic injury associated with CCl₄-induced liver injury.

![FIG. 8. Western blot analysis of CYP2E1 in hepatic microsomes prepared from treated mice. Swiss Webster mice were gavaged with retinol (75 mg/kg, ip) or vehicle for 3 days. On day 6, they were treated with CCl₄ (30 µl/kg, ip). Twenty-four h later, microsomes were prepared as described under Materials and Methods. Microsomal protein (10 µg) was loaded onto each lane of a 10% polyacrylamide gel and immunoblotted with anti-rat CYP2E1 primary antibody (Gentest). Protein was visualized with alkaline phosphatase color reagent and the bands were quantified by scanning densitometry. The positions of the molecular weight markers are indicated on the left (×10³ kDa). The lanes from left to right are retinol + CCl₄ (1–3) vehicle + CCl₄ (4–6), untreated (7), and acetone (8). The arrow represents CYP2E1 and the bottom band is due to a minor cross reactivity with CYP2C11. The bars on the graph represent the mean ± SEM of the optical density for bands from similar treatment groups. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test. *Significantly decreased (p < 0.05) from all other treatment groups. **Significantly increased (p < 0.05) from all other treatment groups.](Image 326x257 to 570x551)

**TABLE 3**

Aniline Hydroxylation Activity following CCl₄ Administration

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>% of untreated control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Acetone</td>
<td>6</td>
<td>375.1 ± 5.3**</td>
</tr>
<tr>
<td>Retinol + CCl₄ (48 h post retinol)</td>
<td>7</td>
<td>96.3 ± 1.1</td>
</tr>
<tr>
<td>Vehicle + CCl₄ (48 h post retinol)</td>
<td>7</td>
<td>54.7 ± 1.4*</td>
</tr>
<tr>
<td>Retinol + CCl₄ (72 h post retinol)</td>
<td>7</td>
<td>46.7 ± 5.3*</td>
</tr>
<tr>
<td>Vehicle + CCl₄ (72 h post retinol)</td>
<td>7</td>
<td>47.1 ± 7.4*</td>
</tr>
</tbody>
</table>

Note. Values are the mean ± SEM. Significance was determined with an ANOVA coupled with the Student-Newman-Keuls post-hoc test.

* Significantly decreased from untreated control, p < 0.05.

** Significantly increased from untreated control, p < 0.01. Untreated control aniline hydroxylation activity was 2.02 ± 0.11 nmol/mg/min.
the metabolite. In the mouse, retinol accomplishes this without altering the constitutive expression of CYP2E1. Future studies will focus on retinol’s ability to compete with CCl4 for CYP2E1 and to analyze CYP2E1 mRNA levels for changes following retinol treatment.

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REFERENCES


