Mechanisms of Circadian Rhythmicity of Carbon Tetrachloride Hepatotoxicity

JAMES V. BRUCKNER, RAGHUPATHY RAMANATHAN,¹ K. MONICA LEE,² and SRINIVASA MURALIDHARA

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, the University of Georgia, Athens, Georgia

Received June 7, 2001; accepted September 21, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The toxicity of carbon tetrachloride (CCl₄) and certain other chemicals varies over a 24-h period. Because the metabolism of some drugs follows a diurnal rhythm, it was decided to investigate whether the hepatic metabolic activation of CCl₄ was rhythmic and coincided in time with maximum susceptibility to CCl₄ hepatotoxicity. A related objective was to test the hypothesis that abstinence from food during the sleep cycle results in lipolysis and formation of acetone, which participates in induction of liver microsomal cytochrome P450IIE1 (CYP2E1), resulting in a diurnal increase in CCl₄ metabolic activation and acute liver injury. Groups of fed and fasted male Sprague-Dawley rats were given a single oral dose of 800 mg of CCl₄/kg at 2- to 4-h intervals over a 24-h period. Serum enzyme activities, measured 24 h post dosing as indices of acute liver injury, exhibited distinct maxima in both fed and fasted animals dosed with CCl₄ near the beginning of their dark/active cycle. Blood acetone, hepatic CYP2E1 activity, and covalent binding of ¹⁴CCl₄/metabolites to hepatic microsomal proteins in untreated rats fed ad libitum followed circadian rhythms similar to that of susceptibility to CCl₄. Parallel fluctuations of greater amplitude were seen in rats fasted for 24 h. Hepatic glutathione levels were lowest at the time of greatest susceptibility to CCl₄. Acetone dose-response experiments showed high correlations between blood acetone levels, CYP2E1 induction, and CCl₄-induced liver injury. Pretreatment with diallyl sulfide suppressed CYP2E1 and abolished the circadian rhythmicity of susceptibility to CCl₄. These findings provide additional support for acetone’s physiological role in CYP2E1 induction and for CYP2E1’s role in modulating CCl₄ chronotoxicity in rats.

Many physiological and biochemical processes in laboratory animals and humans have been found to vary rhythmically over a 24-h period (i.e., to exhibit a circadian or diurnal rhythm). Rhythms are considered as sequences of biological events, repeating themselves in the same order and at the same intervals. Temporal differences in drug action and toxicity have been recognized for over 50 years in humans and in laboratory animals. A number of cyclic physiological processes can affect the absorption, distribution, metabolism, and elimination of drugs and other chemicals (Labrecque and Belanger, 1991).

The chronotoxicity of solvents and other industrial chemicals has received relatively little attention. Volatile organic compounds (VOCs) are a class of solvents to which many people are exposed occupationally and environmentally. Early studies of chloroform (Lavigne et al., 1983), carbon tetrachloride (CCl₄) (Harris and Anders, 1980; Bruckner et al., 1984), and 1,1-dichloroethylene (Jaeger et al., 1975) revealed circadian rhythms in susceptibility of rats to liver damage by the chemicals. Some of the investigators speculated that periods of enhanced susceptibility might be due to cyclic increases in metabolic activation of the VOCs. Temporal variations in P450-catalyzed metabolism of a variety of substrates by rat liver microsomes have been reported, but correlations of substrate metabolism with P450 levels have generally been poor (Labrecque and Belanger, 1991). Miyazaki et al. (1990) described the time dependence of several P450 isoforms active in catalyzing testosterone hydroxylolation in rat liver in a preliminary report. Furukawa et al. (1999) observed high alkoxycoumarin O-dealkylase activities in the liver of rats sacrificed during the animals’ dark cycle but relatively stable total P450 levels. It is possible that

ABBREVIATIONS: VOCs, volatile organic compounds; P450, cytochrome P450; CYP2E1, cytochrome P450IIE1; GSH, glutathione; S-D, Sprague-Dawley; DAS, diallyl sulfide; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; ICD, isocitrate dehydrogenase; AUC, area under the curve; GC, gas chromatograph; NPSH, nonprotein sulfhydryl; GSHPx, glutathione peroxidase; PNP, p-nitrophenol; 4-NC, 4-nitrocatechol; jHB, j-hydroxybutyrate; AA, acetoacetate.
diurnal rhythms in certain P450 isozymes account for the periodicity in xenobiotic metabolism. Unfortunately, very little information relevant to this postulate is available. The P450 substrates used by Furukawa et al. (1999) had low isoform specificity.

Abstinence from food during the sleep cycle may be linked to circadian rhythmicity in CCl4 metabolic activation and acute toxicity. Fasting for 16 to 48 h is known to enhance the metabolism and hepatotoxicity of CCl4 (Harris and Anders, 1980; Nakajima et al., 1982). Fasting has also been found to induce hepatic microsomal cytochrome P450IIE1 (CYP2E1) (Brown et al., 1995). CYP2E1 plays a major role in metabolism of CCl4 and other low molecular weight VOCs (Guengerich et al., 1991; Zangar et al., 2000). It is possible that levels of CYP2E1 vary rhythmically over a 24-h period and that the isozyme’s activity at any given clock time will determine the extent of CCl4 metabolic activation and hepatotoxicity. Fasting can result in significant lowering of hepatic glutathione (GSH) levels. The GSH redox cycle is thought to play a protective role against CCl4-induced oxidative stress by metabolism of hydroperoxides and lipid hydroperoxides (Nishida et al., 1996). Fasting is also known to result in increased circulating levels of ketone bodies, including acetone. CYP2E1 plays a major role in acetone catabolism, as demonstrated by a marked elevation in blood acetone in diallyl sulfide-treated rats (Chen et al., 1994). Conversely, acetone is an inducer of CYP2E1 and certain other P450s (Johansson et al., 1988). Thus, it is possible that lack of food intake during animals’ sleep cycle may trigger the formation of acetone, which can contribute to CYP2E1 induction sufficiently to increase the production of cytotoxic CCl4 metabolites.

Objectives of this investigation were to 1) determine whether acute CCl4 hepatotoxicity and hepatic microsomal CYP2E1 exhibit predictable circadian rhythms in rats maintained on a constant light/dark cycle; 2) test the hypothesis that abstinence from food during sleep results in lipolysis and formation of acetone, which participates in induction of hepatic CYP2E1, resulting in a diurnal rhythm in CCl4 metabolic activation and acute hepatotoxicity; and 3) assess the potential contribution of circadian rhythms in glutathione and glutathione peroxidase activity to CCl4 chronotoxicity. Information gained from this study may be helpful in understanding the synchronization of metabolism of CCl4 and other CYP2E1 substrates.

Materials and Methods

Chemicals. Analytical-grade CCl4 was purchased from J. T. Baker Chemical Co. (Philadelphia, PA). 14C-CCl4 with specific activities of 4 and 61 mCi/mmol was supplied by PerkinElmer Life Sciences (Boston, MA) and Amersharm Pharmacia Biotech (Arlington Heights, IL), respectively. Carbasorb was purchased by Packard Instrument Co. (Downers Grove, IL). All other chemicals and biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Sprague-Dawley (S-D) rats (250–265 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). The animals were housed in their own room in an American Association for the Accreditation of Laboratory Animal Care-approved animal care facility. The experimental protocol was reviewed and approved by an institutional animal care committee. The rats were acclimated to a 12-h light/dark cycle (light 6:00 AM–6:00 PM, i.e., 0600–1800 h) in a temperature (25°C)- and humidity (40%)-controlled room for at least 2 weeks prior to use. Animals were housed in groups of six in stainless steel cages in a ventilated rack. Tap water and food (Purina Rat Chow 5001; Purina, St. Louis, MO) were available ad libitum to some animals. Other groups of rats were fasted for 24 h prior to blood/tissue sampling or CCl4 dosing in experiments requiring fasting. Food was withheld from all animals for 3 h post dosing, but water was provided at all times.

CCl4 Dosing and Blood Sampling. The body weight of animals at the time of experimentation ranged from 320 to 360 g. Appropriate amounts of CCl4 were diluted in corn oil, such that an oral dose of 800 mg of CCl4/kg b.wt. could be given in a total volume of 1.5 ml/kg. Randomized groups of rats were administered a single dose of 800 mg of CCl4/kg by gavage, at 2- to 4-h intervals over a 24-h period. A curved, ball-tipped intubation needle was used to give the oral bolus dose. Controls similarly received 1.5 ml/kg of corn oil. The rats were sacrificed 24 h after CCl4 dosing. Blood was drawn by cardiac puncture, and serum was obtained for assays of enzyme activities.

In a follow-up experiment, nonfasted rats received 20 mg of diallyl sulfide (DAS)/kg p.o. in corn oil 12 h before CCl4. The choice of the 20 mg/kg p.o. dose of DAS was based upon the results of pilot time course and dose-response experiments, in which maximal inhibition of hepatic CYP2E1 activity (65–70% decrease from control) was manifest in rats 12 to 24 h after oral administration of 20 to 40 mg of DAS/kg (data not shown). Groups of the DAS-pretreated rats were gavaged with 800 mg of CCl4/kg in corn oil at 4-h intervals over a 24-h period. Blood was withdrawn by cardiac puncture for serum enzyme measurements 24 h after CCl4 administration.

Serum Enzyme Assays. The activities of three enzymes in serum were monitored as measures of hepatic cellular damage by CCl4. Alanine aminotransferase (ALT), sorbitol dehydrogenase (SDH), and isocitrate dehydrogenase (ICD) activities were measured by standard spectrophotometric techniques. ALT, SDH, and ICD are each expressed as milliunits per milliliter of serum, in which 1 unit of activity is defined as 1 μmol of NAD(H) converted/min/ml of serum.

Acetone Dose-Response Study. The first of a set of experiments was conducted to determine the area under the blood concentration versus time curve (AUC) for each of a series of doses of acetone. An indwelling cannula was surgically implanted into the right carotid artery of rats of 350 to 375 g. The cannula was passed under the skin and exteriorized at the nape of the neck. Food and water were supplied ad libitum during a 24-h recovery period. Each group of the unanesthetized, freely moving animals was given one of the following doses of acetone by corn oil gavage between 9:00 and 10:00 AM: 100, 250, 500, 1000, or 2000 mg/kg. Serial blood samples of 5 to 50 μl were taken at intervals of 2 to 120 min for 8 h post dosing and analyzed for their acetone content as described below.

The dose dependence of hepatic CYP2E1 induction by acetone was evaluated in a second experiment. Groups of six nonfasted rats of ~280 g were given 0, 50, 100, 250, 500, 1000, or 2000 mg of acetone/kg by corn oil gavage. The rats were dosed between 9:00 and 10:00 AM, a time when we found that hepatic CYP2E1 activity was relatively low. The animals were sacrificed 24 h later, so their liver could be processed and microsomal CYP2E1 activity measured as described below.

The objective of the third experiment was to assess the dose dependence of potentiation of CCl4 acute hepatotoxicity by acetone. Groups of nonfasted rats of ~280 g were administered 0, 50, 100, 250, 500, 1000, or 2000 mg of acetone/kg by corn oil gavage between 9:00 and 10:00 AM. After 24 h they were given 800 mg of CCl4/kg by corn oil gavage. The animals were sacrificed 24 h after CCl4 and blood was taken by cardiac puncture for assay of SDH activity, as detailed above.

Acetone Analysis. Blood samples of 5 to 50 μl, taken from an indwelling carotid arterial cannula, were transferred to a 20-ml headspace vial. The vial was immediately capped with a polytetrafluoroethylene-lined septum and tightly crimped. A PerkinElmer model 8500 gas chromatograph (GC) fitted with a flame ionization detector and an HS-101 headspace autosampler was used. Analyses were...
carried out on a 6" × 1/8" stainless steel column packed with 3% OV-17 that was supplied by Alltech Associates (Deerfield, IL). The temperatures for analysis were as follows: column run isothermal at 75°C, injector at 110°C, and detector at 200°C. Nitrogen was used as the carrier gas at 5 psi. The headspace vials were heated at 110°C in the thermostat-controlled autosampler chamber for 30 min before being vented into the GC. Acetone was well resolved without interfering peaks. Standard solutions of acetone in distilled water were analyzed each day as described above to derive a standard curve.

**Liver Assays.** The liver nonprotein sulphydryl (NPSH) content of freshly prepared liver homogenates from undosed rats was measured colorimetrically. Other portions of liver were homogenized in cold 0.02 M Tris-KCl buffer at pH 7.4. Microsomes were prepared by differential centrifugation and stored at −80°C. Glutathione peroxidase (GSHPx) activity in the 105,000g supernatant was assayed according to Lawrence and Burke (1976). The protein concentration and total cytochrome P450 content of hepatic microsomes were determined by conventional spectrophotometric procedures. CYP2E1 activity was estimated by measuring p-nitrophenol (PNP)-hydroxylation. The hydroxylation of PNP to 4-nitrocatechol (4-NC) was determined by the procedure of Koop (1990). A substrate concentration of 100 μM PNP was used in this assay. CYP2E1 activity was expressed as nmol of 4-NC/min/mg of protein.

**Covalent Binding Study.** Groups of undosed rats served as liver donors for a study of covalent binding of 14CCl4 as a function of clock time. Liver samples were taken every 2 to 4 h over a 24-h period. Microsomes were prepared as described previously and diluted with 0.05 M Tris-0.15 M KCl/EDTA buffer (pH 7.4) to a microsomal protein concentration of 2 mg/ml. This preparation was incubated with NADPH (1 mM), MgCl2 (5 mM), nicotinamide (2.5 mM), and 400 nmol/ml 14CCl4 (0.25 μCi/ml). The flasks were capped with latex rubber septa immediately after addition of the 14CCl4 and incubated for 20 min at 37°C. The reaction was stopped with 2 ml of ice-cold ethanol, and the samples were centrifuged in the cold at 1800 g for 15 min. The pellet was washed twice with cold ethanol, dissolved in 0.5 N NaOH, neutralized with 1 N HCl, and counted in a Beckman LS-7500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA) after addition of an appropriate scintillation cocktail. Blanks were prepared using boiled microsomes. The results are expressed as pmol of 14C bound/mg of microsomal protein.

**Safety Precautions.** Studies were conducted in a biohazard facility to avoid CCl4 contamination of laboratory personnel and surrounding areas. Preparation of test solutions, dosing, sacrifices, and blood and tissue sampling were performed under a 100% exhaust hood. The rats were maintained in vented cage racks to avoid exposure of each other and personnel. Animal remains and other CCl4-contaminated materials were sealed in double plastic bags, placed into biohazard barrels, and disposed of by a licensed disposal firm.

14CCl4 was used, monitored for, and disposed of in accordance with university policy.

**Statistical Analyses.** Data were analyzed by one-way analysis of variance. The significance of difference between fed and fasted groups was determined by use of Tukey’s least significant differences test. Area under the blood acetone concentration versus time curve from 0 to 8 h (AUC0–8) values were calculated according to the linear trapezoidal rule. The significance of apparent acetone dose-dependent differences in blood acetone AUC0–8, hepatic CYP2E1 activity, and serum SDH activity were assessed by the Student-Newman-Keuls test using Modstat software by Statistics.Com (Bellingham, WA). The minimum level of statistical significance for these tests was p ≤ 0.05. Correlation coefficients between AUC0–8 and hepatic CYP2E1 activity and between AUC0–8 and serum SDH activity were determined using Prism Version 3.02 software by GraphPad Software (San Diego, CA).

**Results**

**Toxicity.** Rats dosed with 800 mg/kg CCl4 exhibited a pronounced circadian rhythm in susceptibility to the chemical’s hepatotoxic action. The three enzymes used as indices of hepatocellular damage rose and fell in parallel over the 24-h monitoring period (Fig. 1). CCl4 was most hepatotoxic to fasted rats when given at 6:00 PM, the beginning of the animals’ active/dark cycle. The chemical was least hepatotoxic during the middle (i.e., 10:00 AM–2:00 PM) of their inactive/light cycle (Fig. 1A). Serum enzyme levels in these subjects rose very rapidly from a minimum at 10:00 AM to 2:00 PM to a maximum at 6:00 PM. These increases were about 3- to 5-fold. SDH, ALT, and ICD levels did not vary significantly during 24 h in undosed rats (data not shown). It is evident when comparing Fig. 1A with 1B that fasted rats were much more sensitive to CCl4 than fed rats. The diurnal rhythm of cytotoxicity in the fasted animals had a substantially greater amplitude. Maximal hepatocellular injury, as reflected by the serum enzyme levels, however, occurred some 2 h sooner than in the group fed ad libitum. The serum enzyme levels declined more rapidly thereafter in these animals than in their fed counterparts.

As shown in Fig. 1A, DAS pretreatment effectively blocked acute CCl4 hepatotoxicity and abolished the diurnal rhythm in susceptibility to the injury. Serum SDH and ALT activities were similarly affected. The diurnal rhythm in hepatic CYP2E1 activity, which is described below, was also suppressed (data not shown).

**Metabolism.** A circadian rhythm in hepatic microsomal total P450 was not apparent in fed or in fasted rats. The P450 levels were comparable in both groups over the 24-h monitoring period (data not shown). Hepatic microsomal CYP2E1 activity, in contrast to total P450 levels, exhibited a definite circadian rhythm (Fig. 2). CYP2E1 activity in fed rats progressively diminished during the animals’ inactive cycle, reaching its lowest level at 2:00 PM. There was an increase in CYP2E1 between 2:00 and 6:00 PM of ~35% in the fed animals. This interval was followed by stable CYP2E1 activity for the remainder of the active cycle. In contrast, CYP2E1 diminished rapidly during the active cycle, but rose steadily throughout the inactive cycle in the fasted rats, attaining its maximal level at 6:00 PM, the beginning of the active cycle. There was an increase of ~110% during this period (i.e., 6:00 AM–6:00 PM). The isozyme’s activity was significantly higher in the fasted than in the fed rats at all times except 6:00 AM.

The magnitude of covalent binding of 14CCl4/metabolites to hepatic microsomal proteins exhibited a diurnal rhythm (Fig. 3). Binding was lowest during the inactive cycle. An ~80% increase in binding occurred between 6:00 and 8:00 PM in rats fed ad libitum. Thereafter, the level of bound radiolabel remained elevated for the duration of the active period. Covalent binding of 14CCl4 to microsomal proteins of fasted rats also showed a diurnal rhythm, but binding was substantially greater than in the nonfasted animals. There was an abrupt diurnal increase of ~80% in the fasted rats, with the rise occurring some 4 h earlier than in the nonfasted subjects. Binding remained high during the active cycle of the fasted rats.

**Acetone, CYP2E1, and Potentiation of CCl4 Toxicity.** Levels of acetone in the blood exhibited a unique circadian
rhythm (Fig. 4). There was a pronounced peak in the acetone concentration at the beginning of the active cycle in both fed and fasted rats. The blood levels rose abruptly between 2:00 and 6:00 PM and then dropped rapidly during the next 4 h. Blood acetone concentrations were significantly higher throughout the 24-h monitoring period in the fasted animals, particularly at 6:00 PM.

Administration of a series of doses of acetone to rats by corn oil gavage produced dose-dependent increases in blood acetone concentrations, hepatic microsomal CYP2E1 activity, and serum SDH levels. The blood acetone time courses exhibited plateaus rather than true peaks, so AUC$^8_0$ was calculated as the internal dosimeter. The AUC$^8_0$ values were directly proportional to the administered dose of acetone ($\rho^2 = 0.99$). The lowest acetone dosage, 50 mg/kg, induced CYP2E1 and potentiated CCl$_4$ acute hepatotoxicity (Table 1). There were not significant increases in CYP2E1 or SDH activities between 100 and 250 mg/kg, but there were statistically significant dose-dependent elevations in each index in response to 500, 1000, and 2000 mg/kg. There were high degrees of correlation between blood acetone AUC$^8_0$ and CYP2E1 activity ($\rho^2 = 0.96$) and between AUC$^8_0$ and serum SDH activity ($\rho^2 = 0.95$) (Fig. 5, A and B).

Nonprotein Sulphydryls. A prominent diurnal rhythm was seen in the NPSH content of the liver of rats fed ad libitum (Fig. 6). Fasted rats displayed a similar rhythm of lower amplitude. NPSH levels were significantly lower in the fasted animals at each measurement period, particularly at 6:00 PM.

Fig. 1. Circadian rhythmicity of susceptibility of rats to CCl$_4$ hepatotoxicity. Groups of rats were dosed at 2- to 4-h intervals over a 24-h period with 800 mg of CCl$_4$/kg b.wt. SDH, ALT, and ICD activities in serum were measured 24 h after dosing. Each point represents the mean ± S.E. for six animals. For some groups, S.E. bars are too small to be visible. The rats were fed ad libitum (A) or fasted for 24 h (B) before CCl$_4$. ICD activity is not included (in A) for the sake of clarity. SDH levels in rats pretreated with 20 mg of DAS/kg p.o. 12 h before CCl$_4$ are shown instead. As illustrated by the bar parallel to the x-axis, all animals were maintained on a 12-h light/dark cycle with light from 6:00 AM to 6:00 PM (0600–1800 h) and dark from 6:00 PM to 6:00 AM (1800–0600 h).
dark period. There was a lag time of 2 to 4 h in the diurnal rhythm of the fasted rats. Hepatic GSHPx activity did not exhibit a circadian rhythm nor was there a significant difference between fed and fasted animals (data not shown).

Discussion

Findings in the current investigation are consistent with the hypothesis that abstinence from food during the inactive cycle results in lipolysis and formation of acetone, which participates in CCl4 metabolic activation and hepatotoxicity. The susceptibility of male S-D rats to CCl4 hepatotoxicity varies significantly during a 24-h period. The extent of liver damage upon CCl4 exposure during the initial part of the animals' active cycle is substantially greater than that caused by the same dose of chemical given during the inactive cycle. The abrupt increase from minimal to maximal susceptibility consistently occurs just prior to and during the first hours of feeding and other nocturnal activities.

Circadian rhythmicity in metabolic activation of CCl4 is apparently important in the chemical's chronotoxicity in male S-D rats. CCl4 is metabolized by P450-dependent reductive dehalogenation to a trichloromethyl radical. This moiety can covalently bind to lipids and proteins or react further to form organic free radicals, peroxides, and other reactive cytotoxic metabolites. We found covalent binding of
14CCl4 metabolites to hepatic microsomal proteins to be relativ -
atively high in rats during their active cycle. Total P450
levles were relatively constant over a 24-h period in the
current study. In contrast, CYP2E1 activity exhibited a di-
urnal rhythm, increasing significantly (by 6:00 PM), the time
of maximum14CCl4 covalent binding and toxicity. CYP2E1 is
the principal P450 isozyme that catalyzes the metabolism of
CCl4 and other VOCs in rodents and humans (Guengerich et
al., 1991; Wong et al., 1998; Zangar et al.,
2000). In the
current study, pretreatment of rats with DAS, a CYP2E1
inhibitor, negated the rhythmicity of CYP2E1 activity and
CCl4 cytotoxicity, demonstrating the central role of CYP2E1
in this phenomenon.

Circadian variations in CCl4 metabolism and toxicity ap -
ppear to be related to restricted food intake during inactive/
sleep periods, because fasting is known to potentiate CCl4
hepatotoxicity. Nakajima et al. (1982), for example, observed
a 3.3-fold increase in CCl4 metabolism and a marked eleva-
tion of serum enzymes in male Wistar rats fasted for 24 h
before CCl4 exposure. Brown et al. (1995) reported time-
dependent CYP2E1 increases in male F-344 rats fasted for 8,
16, and 24 h. We observed an increase in CYP2E1 activity of
~35% between 2:00 and 6:00 PM and ~50% between 2:00 PM

<table>
<thead>
<tr>
<th>Acetone Dosage (mg/kg)</th>
<th>AUC0-8 (mg · min/ml)</th>
<th>CYP2E1 (nmol 4-NC/min/mg protein)</th>
<th>SDH Activity (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N.D.</td>
<td>0.9 ± 0.04a</td>
<td>17 ± 7a</td>
</tr>
<tr>
<td>50</td>
<td>N.D.</td>
<td>1.2 ± 0.1b</td>
<td>64 ± 14b</td>
</tr>
<tr>
<td>100</td>
<td>64 ± 3a</td>
<td>2.1 ± 0.1c</td>
<td>162 ± 25c</td>
</tr>
<tr>
<td>250</td>
<td>108 ± 9a</td>
<td>2.2 ± 0.1c</td>
<td>196 ± 42c</td>
</tr>
<tr>
<td>500</td>
<td>227 ± 10b</td>
<td>3.1 ± 0.3c</td>
<td>406 ± 72c</td>
</tr>
<tr>
<td>1000</td>
<td>456 ± 32c</td>
<td>4.5 ± 0.2c</td>
<td>1257 ± 124c</td>
</tr>
<tr>
<td>2000</td>
<td>983 ± 78d</td>
<td>5.5 ± 0.1d</td>
<td>1887 ± 107d</td>
</tr>
</tbody>
</table>

N.D., not determined.

a–f Values for each index that are significantly different from one another at p ≤ 0.05 are designated by a different letter.

14CCl4 metabolites to hepatic microsomal proteins to be rela-
tively high in rats during their active cycle. Total P450
levels were relatively constant over a 24-h period in the
current study. In contrast, CYP2E1 activity exhibited a di-
urnal rhythm, increasing significantly (by 6:00 PM), the time
of maximum14CCl4 covalent binding and toxicity. CYP2E1 is
the principal P450 isozyme that catalyzes the metabolism of
CCl4 and other VOCs in rodents and humans (Guengerich et
al., 1991; Wong et al., 1998; Zangar et al., 2000). In the
current study, pretreatment of rats with DAS, a CYP2E1
inhibitor, negated the rhythmicity of CYP2E1 activity and
CCl4 cytotoxicity, demonstrating the central role of CYP2E1
in this phenomenon.

Circadian variations in CCl4 metabolism and toxicity ap -
ppear to be related to restricted food intake during inactive/
sleep periods, because fasting is known to potentiate CCl4
hepatotoxicity. Nakajima et al. (1982), for example, observed
a 3.3-fold increase in CCl4 metabolism and a marked eleva-
tion of serum enzymes in male Wistar rats fasted for 24 h
before CCl4 exposure. Brown et al. (1995) reported time-
dependent CYP2E1 increases in male F-344 rats fasted for 8,
16, and 24 h. We observed an increase in CYP2E1 activity of
~35% between 2:00 and 6:00 PM and ~50% between 2:00 PM

Fig. 4. Circadian rhythm of blood acetone levels in fasted and non-
fasted rats. Six rats from each group were sacrificed every 4 h over a 24-h
period. Blood acetone concentrations were measured by headspace gas
chromatography. Points represent mean ± S.E. S.E. bars are too small
to be visible for some groups. All values for the fasted groups are signif-
ically higher than corresponding values for the nonfasted groups at
p < 0.05.

Fig. 5. A, correlation between area under the blood acetone concentra-
tion versus time curves from 0 to 8 h post-acetone dosing (AUC0-8) and hepatic
microsomal CYP2E1 activity. Best linear regression (solid line): y = 0.0074X ± 1.27, r² = 0.96, p = 0.004, n = 6. B, correlation between AUC0-8 and serum SDH activity. Best linear regression (solid line): y = 2.696X + (-47.63), r² = 0.95, p = 0.004, n = 6.
and 6:00 AM in rats fed ad libitum. Fasting, stress, and exercise each stimulate lipolysis to satisfy the body’s need for energy. Excess fatty acids can be converted to β-hydroxybutyrate (βHB) and acetoacetate (AA), the latter undergoing decarboxylation to acetone. Acetone is a potent inducer of CYP2E1 and CCl₄ metabolism (Johansson et al., 1988). Acetone, a substrate for CYP2E1, is believed to induce CYP2E1 primarily by binding to it and thereby protecting it from cAMP-dependent phosphorylation (Ronis et al., 1991). Acetone formation during maximal lipolysis each day likely contributes to diurnal induction of CYP2E1. Mlekusch (1982) and Ahlersova et al. (1985) observed sharp peaks in plasma AA and βHB like that we saw for acetone at the onset of the active cycle of rats. We observed that CYP2E1 activity and covalent binding of 14CCl₄ were also maximal or near-maximal during this time. Clark and Powis (1974) reported a peak in aniline hydroxylase activity within 30 min of i.p. injection of female rats with acetone. Such a rapid increase is compatible with the mechanism of ligand stabilization of CYP2E1.

It was of interest in the current study that blood acetone levels diminished so rapidly after reaching their zenith, but CYP2E1 activity remained high. The acetone bound to CYP2E1, however, is not measured by our analytical GC headspace technique. As noted previously, the binding prolongs the residence time of the CYP2E1. After metabolism of its substrate (i.e., acetone), CYP2E1 is degraded quickly. CYP2E1 has a half-life of 4 to 6 h or less in rat liver (Roberts et al., 1994). Ingested CCl₄ reaches the liver, undergoes metabolic activation, and damages the liver in a matter of minutes (Rao and Recknagel, 1968; Sanzgiri et al., 1997). Thus, there appears to be time during a 24-h period for the necessary sequence of events to occur and to be reversed for subsequent diurnal cycles.

Acetone plays an important role in potentiation of CCl₄ hepatotoxicity in fasted subjects. Sato and Nakajima (1985) found that a 1-day fast enhanced the metabolism of CCl₄ and seven other VOCs in male Wistar rats. If acetone is a mediator of fasting-induced CYP2E1 increase and ensuing CCl₄ metabolic activation/toxicity, the magnitude of each process should vary directly with the duration of fasting. Miller and Yang (1984), indeed, reported reasonably good correlation between the duration of fasting and blood acetone and between acetone and hepatic N-nitrosodimethylamine demethylation activity in male S-D rats. Charbonneau et al. (1986) correlated acetone dose/blood level and CCl₄ hepatotoxicity in male S-D rats but did not examine relationships among acetone, CYP2E1, and CCl₄ toxicity. Our findings of substantially higher blood acetone, hepatic CYP2E1, 14CCl₄ covalent binding, and serum enzymes in fasted rats rather than in nonfasted rats were anticipated. It was surprising, however, that circadian rhythms in the two sets of animals generally paralleled one another. Although we did not monitor beyond one 24-h fasting period, other researchers have found diurnal rhythms in lipid metabolites to persist during an additional 24 to 48 h of fasting (Ahlersova et al., 1985; Escobar et al., 1998).

Acetone may not be formed in sufficient amounts during the rat’s 12-h inactive period to account solely for diurnal CYP2E1 induction. Concentrations of AA and βHB are typically higher than acetone levels, so these two ketone bodies could augment CYP2E1 induction by acetone. Results of limited in vivo (Barnett et al., 1992) and in vitro (Zangar and Novak, 1998) studies indicate that neither βHB nor AA induces CYP2E1. Johansson et al. (1988) demonstrated that starvation for 3 days exerted a synergistic effect with acetone on induction of CYP2E1 and CCl₄ metabolism. These researchers believed that this synergistic effect of fasting could be the result of an increased rate of CYP2E1 gene transcription or mRNA stabilization, possibly due to action of stress hormones. Plasma levels of a number of hormones, including
insulin and corticosterone (Mlekusch, 1982), exhibit circadian rhythms with peaks that coincide in time with the maximum CYP2E1 activity and CCl_4 toxicity we observed. Corticosterone and insulin are active in lipolysis and ketogenesis, although they and other hormones may also be involved at the level of the gene in regulation of CYP2E1. Growth hormone plays a major modulatory role by suppressing CYP2E1 gene transcription in S-D rat liver (Chen et al., 1999). It is not clear whether growth hormone participates in the CYP2E1 diurnal cycle that we observed, because growth hormone secretion in male rats is pulsatile (i.e., every 3–4 h), and apoprotein synthesis requires a period of hours.

Certain factors in addition to CYP2E1 activity may contribute to the diurnal rhythm in CCl_4 hepatotoxicity. Liver blood flow is somewhat higher during periods of physical activity. The net effect of a higher blood flow on CCl_4 metabolism should be modest, however, due to the relatively high dose we administered and the slow metabolism of CCl_4 (Gar-gas et al., 1986). In the current study, there was a pronounced circadian rhythm in liver NPSH (i.e., primarily GSH), with levels lowest at the time of maximal susceptibility to CCl_4 cytotoxicity. A similar phenomenon has been reported for chloroform (Lavigne et al., 1983), acetaminophen (Schnell et al., 1983), and 1,1-dichloroethylene (Jaeger et al., 1973). Although GSH conjugation plays a major role in detoxification of the latter two compounds, this is not the case for CCl_4. The formation of a CCl_4-derived free radical adduct with GSH has been demonstrated in vitro (Connor et al., 1990), but this interaction is not believed to be quantitatively important in vivo. GSH does impede CCl_4-induced lipid peroxidation by GSHPx-catalyzed metabolism of hydrogen peroxide and lipid peroxides (Harisch and Meyer, 1985; Nishida et al., 1996). The latter group of investigators found that the GSH redox cycle protected against early stages of hepatocellular injury and liperoxidation but not against more pronounced changes 12 to 24 h after i.p. injection of mice with 1.6 g of CCl_4/kg. Harris and Anders (1980) have demonstrated that marked (i.e., 83%) depletion of liver GSH by diethyl maleate potentiated CCl_4 hepatotoxicity in S-D rats. The diurnal NPSH decreases we observed (i.e., ~30%) were less pronounced. Mitchell et al. (1985) observed marked enzyme leakage from primary cultures of rat hepatocytes when GSH was reduced below a threshold of 20% of control, irrespective of the chemical used to deplete GSH. Harris and Anders (1980) noted that fasted rats exhibited liver injury that was twice as severe as their diethyl maleate-potentiated counterparts, indicative of involvement of other factors. Our own results demonstrate that CYP2E1 induction is an important factor in fasting-elicited and in diurnal changes in susceptibility of rats to CCl_4. The relatively modest decrease in liver NPSH that we saw would appear to be a less important diurnal variable than CYP2E1 induction, although it is not possible to determine their relative contribution from findings in the current investigation.

Findings with rats in the current investigation may be directly relevant to humans. Chronological variations in the pharmacokinetics of nicotine (Gries et al., 1996), ethanol (Sturtevant et al., 1978), aminopyrine (Poley et al., 1978), and a variety of other well metabolized drugs have been reported in human subjects. Interestingly, maximum clearance rates are usually observed during the first part of the subjects’ active cycle. Ketone bodies in the blood exhibit diurnal variations and exhalation of acetone peaks before breakfast (Wildenhoff, 1972). Thus, people may be more susceptible to CCl_4 and other chemicals that undergo CYP2E1-mediated activation when exposed to the chemicals during their initial waking hours.

Information gained from the current study of the circadian rhythmicity of CCl_4 hepatotoxicity may have a number of applications. The phenomenon may well apply to a variety of VOCs and other lipophilic, low molecular weight compounds that are metabolically activated by CYP2E1 to cytotoxic or mutagenic metabolites. The toxic and carcinogenic potency of such chemicals could vary with the (clock) time of exposure. Thus, such chemicals should be evaluated at the time(s) of maximum sensitivity of the test animal. Light/dark cycles and treatment times should be considered in designing experimental protocols. P450 isozymes other than CYP2E1 may also be found to exhibit diurnal rhythms.

Acknowledgments

We appreciate the efforts of Libby Moss and Mary Eubanks in preparation of the manuscript.

References


**Address correspondence to:** Dr. James V. Bruckner, The University of Georgia, College of Pharmacy, Department of Pharmaceutical and Biomedical Sciences, D. W. Brooks Drive, Athens, GA 30602-2352. E-mail: bruckner@rx.uga.edu