DIET-RESTRICTED RATS

SHASHI K. RAMAIAH, UDAYAN APTE, AND HARIHARA M. MEHENDALE

Department of Toxicology and Louisiana Institute of Toxicology, College of Pharmacy and Health Sciences, University of Louisiana at Monroe, Monroe, Louisiana

(Received December 4, 2000; accepted April 18, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

Earlier studies have shown highly exaggerated mechanism-based liver injury of thioacetamide (TA) in rats following moderate diet restriction (DR) and in diabetes. The objective of the present study was to investigate the mechanism of higher liver injury of TA in DR rats. Since both DR and diabetes induce CYP2E1, we hypothesized that hepatic CYP2E1 plays a major role in the bioactivation-based liver injury of TA. When male Sprague-Dawley rats (250–275 g) were maintained on diet restriction (DR, 35% of ad libitum fed rats, 21 days) the total hepatic microsomal cytochrome P450 (CYP450) was increased 2-fold along with a 4.6-fold increase in CYP2E1 protein, which corresponded with a 3-fold increase in CYP2E1 activity as measured by chlorzoxazone hydroxylation. To further test the involvement of CYP2E1, 24 and 18 h after pretreatment with pyridine (PYR) and isoniazid (INZ), specific inducers of CYP2E1, male Sprague-Dawley rats received a single administration of 50 mg of TA/kg (i.p.). TA liver injury was >2.5- and >3-fold higher at 24 h in INZ + TA and TA + INZ groups, respectively, compared with the rats receiving TA alone. Pyridine pretreatment resulted in significantly increased total CYP450 content accompanied by a 2.2-fold increase in CYP2E1 protein and 2-fold increase in enzyme activity concordant with increased liver injury of TA, suggesting mechanism-based bioactivation of TA by CYP2E1. Hepatic injury of TA in DR rats pretreated with diallyl sulfide (DAS), a well known irreversible in vivo inhibitor of CYP2E1, was significantly decreased (60%) at 24 h. CCl₄ (4 ml/kg i.p.), a known substrate of CYP2E1, caused lower liver injury and higher animal survival confirming inhibition of CYP2E1 by DAS pretreatment. The role of flavin-containing monooxygenase (FMO) in TA bioactivation implicated by previous in vitro studies, and consequent increased TA-induced liver injury in DR rats was tested in vivo with a relatively selective inhibitor of FMO, indole-3-carbinol, and then treated with 50 mg of TA/kg. FMO activity and alanine aminotransferase levels measured at different time points revealed that TA liver injury was not decreased although FMO activity was significantly decreased, suggesting that hepatic FMO is unlikely to bioactivate TA. These findings suggest induction of CYP2E1 as the primary mechanism of increased bioactivation-based liver injury of TA in DR rats.

Diet is one of the key factors of our environment that has substantial impact on human health. The close association between diet and xenobiotic metabolism may be traced back to prehistoric days in “animal-plant warfare” during evolution (Chung et al., 1992). Plants synthesized chemicals for self-protection and animals had to develop xenobiotic metabolizing enzymes such as cytochrome P450s for the detoxification of these chemicals. It is well known that various dietary factors have marked effects on the metabolism of drugs, environmental chemicals, and certain endogenous substrates (Chung et al., 1992). Diet or caloric restriction is known to promote good health (Leakey et al., 1998). Diet restriction is known to markedly decrease the acute toxicity of an antiviral agent, ganciclovir (Berg et al., 1994), and isoproterenol (Duffy et al., 1995). Earlier studies have shown that moderate diet restriction has a decisive impact on the final outcome of hepatotoxicity inflicted by thioacetamide (TA) (Ramaiah et al., 1998a, 2001). Although diet restriction led to marked increase in liver injury of thioacetamide, paradoxically, 70% survival was observed from an ordinarily lethal dose of thioacetamide (Ramaiah et al., 1998a, b, 2001).

Thioacetamide, originally used as a fungicide, is a potent hepatotoxicant. Studies have shown that this compound is bioactivated by CYP450 and/or flavin-containing monooxygenase (FMO) systems to sulfine (sulfoxide) and sulfene (sulfone) metabolites, which cause centrilobular necrosis (Hunter et al., 1977; Porter et al., 1979). Studies suggest that thioacetamide sulfoxide, a relatively stable intermediate
of thioacetamide metabolism, is obligatory for the hepatotoxic effects of this compound (Fig. 1), indicating that it is the penultimate reactive metabolite (Porter and Neal, 1978; Porter et al., 1979). Accordingly, it has been reported that the hepatotoxic effects of thioacetamide are only expressed after metabolic conversion to thioacetamide S-oxide that undergoes further metabolic conversion to an as yet unidentified metabolite, probably the reactive unstable thioacetamide sulfone (Hunter et al., 1977; Porter and Neal, 1978; Porter et al., 1979). Since much of the TA bioactivation mechanism and toxicity work was conducted largely prior to the advent of the discovery of CYP450 isozymes, information on specific isozymes involved in the bioactivation of TA has remained completely blurred and uninvestigated, until recently, when Wang et al. (2000b) reported that TA bioactivation is primarily mediated by hepatic CYP2E1.

The objective of this study was to investigate the mechanism of the highly increased liver injury of TA in moderate diet restriction. CYP2E1 is a major constitutive enzyme of mammalian liver with critical roles in xenobiotic metabolism, toxicity, and carcinogenesis (Ronis et al., 1996). Tissue levels of this isozyme are induced by exposures to a diverse group of chemicals such as acetone, benzene, ethanol, isoniazid, and pyridine and certain pathophysiological states such as starvation, diabetes, and obesity (Johansson and Ingelman-Sundberg, 1988; Kim et al., 1988; Park et al., 1993). Hepatic CYP2E1 expression is also markedly influenced by nutritional factors (Chung et al., 1992; Qu et al., 1998; Wang et al., 2000b). We have previously reported that moderate diet restriction (DR) substantially increases liver injury of TA (Ramaiah et al., 1998a,b, 2001). Mechanism-based liver injury of TA is also highly augmented in diabetic condition (Wang et al., 2000a). Other isoforms of CYP450 such as CYP1A1 and CYP2B1/2 are unlikely to be involved: El-Hawari and Plaa (1983) reported that neither induction by 3-methyl cholanthrene (CYP1A1) nor by phenobarbital (CYP2B1/2) increased liver injury of TA. Since both DR and diabetes are known to be accompanied by induced CYP2E1, we hypothesized that hepatic CYP2E1 plays a major role in the bioactivation of TA. We report here that CYP2E1 plays a major role in the mechanism-based liver injury of TA. Furthermore, increased mechanism-based liver injury of TA in DR rats is due to increased bioactivation of TA via induction of CYP2E1. FMO is another family of monooxygenases involved in the oxidation of many sulfur-containing compounds and other soft nucleophiles. FMO has been implicated in the bioactivation of TA (Tyner and Hodgson, 1983; Venkatesh et al., 1991). In vivo and in vitro experiments designed to test the involvement of FMO in the increased TA bioactivation and consequent increased liver injury clearly revealed that FMO is highly unlikely to play a role in increased TA liver injury in DR rats.

**Materials and Methods**

**Chemicals.** Thioacetamide, pyridine, isoniazid, diallyl sulfide, and carbon tetrachloride were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive chlorzoxazone ([4,14C], 40 mM in acetonitrile with 25 μCi/ml) was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Glucose-6-phosphate dehydrogenase was obtained from Calbiochem (La Jolla, CA). All other biochemicals and chemicals were of the highest commercially available quality.

**HPLC System.** All HPLC solvents were passed through an 0.45-μm Millipore membrane and degassed prior to use. HPLC analysis was carried out according to the reported procedure (Peter et al., 1990). Briefly, analyses were performed using a gradient instrument system of Waters HPLC Module 1 with 50% split on Radiometric Flo-Detector using Ultima Flo-M at 2.1 HPLC fluid containing 100% methanol and 5 mM KH2PO4 in 5% methanol. Chlorozoxazone hydroxylation was analyzed on a Supelco C18 250-× 4.6-mm column. The flow rate was 1.5 ml/min. The effluent was monitored at 287 nm. The Rf value of chlorozoxazone and 6-OH-chlorozoxazone was 2.4 and 7.6 min, respectively.

**Animals and Diet Regimens.** Male Sprague-Dawley rats (250–275 g) were maintained in our central animal facility. They were housed individually over sawdust bedding known to be free of any chemical contamination. The ad libitum group had free access to water and normal rodent chow (Harlan Teklad Rat Chow 7001, Madison, WI); protein 25%, fat 4.25%, fiber 4.67%, vitamins and minerals supplemented, calories 3.94 kcal/g at all times. The food consumption of these rats was measured daily for a period of 21 days and then averaged to calculate the mean consumption of food per gram of body weight. The rats in the DR group were allowed 65% of the ad libitum (AL) daily food consumption for a period of 21 days and maintained on the same regimen after TA or vehicle injection. Under these conditions DR rats eat all the food made available to them. The diet restriction protocol used in this study has been described in detail previously (Ramaiah et al. 1998a,b, 2001). Clinical observations of systemic toxicity, weight gain, and food consumption were recorded on a weekly basis.

**Pretreatments. CYP2E1 induction studies.** One group of AL rats received i.p. injection of pyridine (200 mg/kg) or isoniazid (250 mg/kg) in saline (1 ml/kg). The other AL group received only saline vehicle (1 ml/kg). Twenty-four hours (PYR) and 18 h (INZ) later these rats received a single i.p. injection of thioacetamide (50 mg/kg) dissolved in normal saline (0.9% NaCl, 1 ml/kg). The respective controls received saline (1 ml/kg) as vehicle at the time of TA injection.

**CYP2E1 inhibition studies.** Rats maintained on DR regimen were used and treated as follows. Group I (Veh + TA) received corn oil (1 ml/kg) 12 h prior to a single injection of TA (50 mg/kg i.p.). Group II (DAS + TA) received a single injection of diallyl sulfide (200 mg/kg in corn oil, 1 ml/kg) 12 h prior to the administration of TA as in group I. Group III (DAS + Veh) received diallyl sulfide as in group II and saline as vehicle instead of TA injection.

**CCl4 lethality studies to corroborate CYP2E1 inhibition.** CCl4 (4 ml/kg i.p.), a known substrate of CYP2E1, was used to corroborate inhibition of CYP2E1 by DAS. One experiment was designed to determine whether lethality of CCl4 (4 ml/kg i.p.) is decreased by DAS. One group of rats (n = 5; group I) received an i.p. injection of corn oil (1 ml/kg) 12 h prior to a single lethal dose of CCl4 (4 ml/kg i.p. as a 1:1 solution in corn oil), and the other group (n = 5; group II) received a single dose of diallyl sulfide (200 mg/kg in corn oil, 1 ml/kg) 12 h prior to CCl4 injection. These rats were observed twice daily for 14 days for survival/mortality results and cumulative mortality was recorded and expressed as percent mortality.

**Plasma Enzymes.** Blood was collected in heparinized tubes from the dorsal aorta of rats (n = 4 for each time point) under diethyl ether anesthesia at various times after TA or vehicle administration, and the plasma was separated by centrifugation for the estimation of alanine aminotransferase (ALT; EC 2.6.1.2.) as marker of liver injury using Sigma kit 59 UV (ALT) (Sigma Chemical Co.).

**Preparation of Microsomes.** AL and DR rats were weighed, anesthetized using diethyl ether, and necropsied. Individual livers were perfused in situ with ice-cold 0.9% saline, rapidly excised, blotted dry, weighed, minced, and homogenized (1:5 w/v) in ice-cold Tris-acetate buffer (pH 7.4) containing 1.15% KCl. The homogenate was centrifuged at 10,000g for 30 min at 4°C. The supernatant was then centrifuged at 100,000g for 60 min at 4°C. Microsomal pellets were resuspended by homogenization in a Potter Elvehjem glass homogenizer, and again centrifuged at 100,000g. Microsomal pellets were again recovered, quick frozen, and stored at −70°C for later use.

**Western Blot Analysis for CYP2E1.** Western blot analysis of CYP2E1 was carried out as previously described by Wang et al. (2000b). Microsomal...
protein (20 μg) from AL, DR, and with and without pyridine treatment was separated on SDS-polyacrylamide gel and was transferred to nitrocellulose membrane. The membrane was incubated with a rabbit anti-rat CYP2E1 antibody (a generous gift from Dr. Magnus Ingelman-Sandberg, Karolinska Institute, Stockholm, Sweden) and further probed with donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The blots were visualized using enhanced chemiluminescence kit by Pierce (Rockford, IL) and densitometric analysis was carried out using a GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

Enzyme Assays. Frozen microsomes from each animal were suspended in 50 mM Tris-acetate buffer (pH 7.4) by homogenization (Potter Elvehjem) and microsomal protein was estimated (Lowry et al., 1951) using bovine serum albumin as standard. Microsomal CYP450 and cytochrome b$_{5}$ content were estimated according to the established procedure by the CO difference spectrum method (Omura and Sato, 1964) and expressed as nanomoles per milligram of protein. Chlorzoxazone-6-hydroxylation assay (using 4-14 C-labeled chlorzoxazone) was used to estimate CYP2E1 activity (Peter et al., 1990) and expressed as nanomoles of CHZ/min/mg of protein. A final concentration of 800 μM chlorzoxazone was incubated in (0.1 M potassium phosphate buffer, pH 7.4) with liver microsomes (containing 200–400 μM of microsomal protein) and an NADP+-generating system (consisting of 50 mM glucose-6-phosphate, 50 mM MgCl$_{2}$ made up in double distilled water, 15 mM NADP made up in phosphate buffer, 100 IU/ml glucose-6-phosphate dehydrogenase in phosphate buffer) in a total volume of 0.1 ml. Chlorzoxazone working solutions were freshly prepared. Incubations were started by the addition of the NADP+-generating system. After 30 min the reactions were quenched by the addition of 2 ml of methylene chloride. Incubations were extracted twice with 1 ml of methylene chloride by shaking in capped vials, using a mechanical mixing device. The layers were separated by centrifugation at 2500 g for 5 min, and the combined organic layers were evaporated to dryness under nitrogen at ambient temperature with an N-EVAP analytical evaporator (Organamation Associates Inc., Berlin, MA). Residues were dissolved in 70 μl of methanol in a Wisp vial and used for HPLC analysis. The effluent was monitored at 287 nm. Recovery of 6-OH-CHZ was 74 ± 4% and total activity was left uncorrected. To calculate chlorzoxazone hydroxylase activity, as a measure of CYP2E1 activity, specific activity of chlorzoxazone stock solution, disintegrations per minute of 50-μl aliquots of the chlorzoxazone working solution, and protein concentration were used.

In Vivo Exposure to Indole-3-Carbinol (I3C) and TA Toxicity. I3C, a known in vivo inhibitor of FMO, was used to assess the contribution of FMO in TA-induced liver injury. To investigate the involvement of FMO1 in TA-induced liver injury, an FMO1 inhibition study was conducted with AL and DR rats. I3C, an in vivo inhibitor of FMO (Larsen-Su and Williams, 1996), was mixed in powdered rat chow at a concentration of 0.25% (w/w). AL and DR rats were maintained on this diet during the last 10 days of their 21-day dietary protocol. At the end of their 21 day dietary protocol these rats received a low dose of TA (50 mg/kg). Plasma ALT was measured as a marker of liver injury over a time course (0–120 h) after TA administration in the AL group. Results are expressed as mean ± S.E.M. (n = 4). * values significantly different from the AL group at the corresponding time point.

Results

Body Weight Gain and Liver Weight. The body weight gain was measured twice weekly in AL and DR rats. There was a significant reduction (66%) in body weight gain in DR rats compared with AL group. The liver weights were measured at the end of 21 days. Liver weight of DR rats decreased by 52% (P ≤ 0.01). To determine whether increased total CYP450 content and CYP2E1 activity in DR rats was an artifact due to decrease in liver weight, protein levels were compared per gram of liver. No significant difference was found when protein levels were expressed as microsomal protein (milligram per gram of liver weight) in AL and DR rats (data not shown).

Effect of Inducers of CYP2E1 on Thioacetamide Liver Injury. If CYP2E1 is the mediator of TA bioactivation and mechanism-based liver injury of TA then inducers of CYP2E1 such as PYR and INZ should increase TA liver injury. Plasma ALT was estimated as marker of liver injury over a time course following TA (50 mg/kg i.p.) administration. This dose was used, since 50 mg of TA/kg caused 4%-fold higher liver injury in DR rats compared with AL rats (Ramaiah et al., 1998b). Figure 2 shows plasma ALT activity at various time points following TA administration after pyridine pretreatment. ALT activity was increased 2.5-fold at 24 h in PYR + TA group compared with Veh + TA group at 24 h at 36 h in the Veh + TA group. Isoniazid is known to be a more specific in vivo inducer of CYP2E1 (Park et al., 1993). Liver injury of TA was increased 3.5-fold in INZ + TA group compared with Veh + TA group at 24 h at various time points (Fig. 3).

Cytoskeleton P450 Content, CYP2E1 Protein, and CYP2E1 Activity. To ascertain the induction of CYP2E1 by PYR, total hepatic microsomal cytosome P450 content and CYP2E1 activity were determined following PYR treatment prior to as well as following TA administration. There was a significant increase in total P450 content and CYP2E1 levels at 0 h in the PYR + TA group (Fig. 4, A and B), confirming induction of CYP2E1 isozyme by PYR. This was followed by a 2-fold increase in total CYP450 content and a 2-fold increase in CYP2E1 activity at 24 h after TA injection (Fig. 4, A and B), Western
Blot analysis of hepatic microsomal proteins from PYR-treated rats revealed a 2.2-fold induction of CYP2E1 protein (Fig. 5). Since DR resulted in a 6-fold increase in liver injury following 50 mg of TA/kg i.p., total CYP450 and specific CYP2E1 levels were estimated in DR and AL rats following TA administration at this same dose. DR alone resulted in a 2-fold higher total CYP450 content and 3-fold increase in CYP2E1 activity (Fig. 6, A and B). Western blot analysis of hepatic microsomal proteins from rats maintained on AL and DR regimens revealed a 4.6-fold increase in CYP2E1 protein in DR rats (Fig. 7). No further increase in either total CYP450 content and/or CYP2E1 activity was evident in DR rats 24 h following TA injection. In the AL rats both total CYP450 and CYP2E1 were elevated 2-fold at 24 h after TA administration (Fig. 6, A and B), suggesting that TA is an inducer of CYP2E1.

Additionally, we estimated cytochrome b5 activity to investigate whether increased liver injury of TA is due to induction of another enzyme. No significant change in cytochrome b5 activity following DR alone or after treatment with thioacetamide was evident (data not shown), suggesting that increase in CYP2E1 in DR and in AL rats after TA treatment is a relatively specific effect.

**Effect of in Vivo Inhibition of CYP2E1 on Hepatotoxicity of TA.** The objective of this study was to investigate whether pretreatment of rats with DAS, a known in vivo irreversible inhibitor of CYP2E1, would yield decreased liver injury of TA in DR rats. A decrease in liver injury would be consistent with the hypothesized role of CYP2E1 in the bioactivation of TA and the mechanism-based infliction of liver injury. Liver injury of TA was decreased by 60% in the DR rats pretreated with diallyl sulfide (Fig. 8). Markedly decreased liver injury was confirmed by histopathological changes observed in liver sections under a light microscope (data not shown). These results further support the involvement of CYP2E1 in the higher liver injury of TA in DR rats.

**Lethality Studies to Confirm CYP2E1 Inhibition.** To ascertain the inhibition of CYP2E1 by in vivo treatment with DAS, CCl4 toxicity and lethality study was conducted in rats pretreated with DAS. CCl4 was used to assess the in vivo activity of CYP2E1, an obligatory bioactivator of CCl4 known to be essential for hepatonecrob
genecity of CCl₄ (Wong et al., 1998). Figure 9 represents liver injury of CCl₄ upon pretreatment with DAS. As expected, administration of CCl₄ (4 ml/kg i.p.) alone resulted in high liver injury as assessed by plasma ALT activity at 12 h. However, in the group pretreated with DAS, CCl₄ caused a 6-fold decrease in liver injury. Between 12 and 24 h after CCl₄ injection there was 100% mortality in the rats not receiving DAS pretreatment, while 50% survival occurred in the DAS-pretreated group (data not shown), confirming the in vivo inhibition of CYP2E1 by DAS. Because of 100% mortality in the group not pretreated with DAS, plasma ALT values are unavailable for the 24-h time point in Fig. 9. These results (Figs. 8 and 9) collectively confirm that CYP2E1 plays a role in the bioactivation of TA in inflicting mechanism-based liver injury. Furthermore, these findings also suggest that highly increased mechanism-based TA liver injury in DR rats is due to DR-induced CYP2E1.

FMO Activity and Liver Injury Following I3C Administration. If FMO is involved in the bioactivation of TA, in vivo inhibition of FMO should result in decreased TA-induced liver injury. Dietary administration of I3C for 10 days decreased FMO1 activity in both AL and DR rats (Fig. 10). In AL rats hepatic FMO activity was reduced by 50% and in DR rats it was reduced by 33% (Fig. 10). However, TA-induced liver injury was substantially increased in the AL + I3C-treated rats at 24 h (Fig. 11) and in the DR + I3C-treated rats the change was not significant (Fig. 11). These results show that FMO inhibition by I3C does not result in decreased liver injury, suggesting that FMO is highly unlikely to be associated with increased TA liver injury. Indeed, liver injury was increased in AL rats with FMO inhibition, suggesting that hepatic FMO may be involved in the detoxification of TA. Because of lower FMO, a corresponding incremental amount of TA would become available for bioactivation by CYP2E1. It is possible that higher availability of TA for bioactivation by CYP2E1 may explain the higher liver injury.

Discussion
Earlier studies have shown that DR substantially increases mechanism-based liver injury of TA. Liver injury was >6-fold higher at a low dose (50 mg/kg i.p.) and >2.5-fold higher at lethal dose of TA (600 mg/kg i.p.) in DR rats compared with their AL cohorts (Ramaiah et al., 1998). Figure 6 represents liver injury of CCl₄ upon pretreatment with DAS. As expected, administration of CCl₄ (4 ml/kg i.p.) alone resulted in high liver injury as assessed by plasma ALT activity at 12 h. However, in the group pretreated with DAS, CCl₄ caused a 6-fold decrease in liver injury. Between 12 and 20 h after CCl₄ injection there was 100% mortality in the rats not receiving DAS pretreatment, while 50% survival occurred in the DAS-pretreated group (data not shown), confirming the in vivo inhibition of CYP2E1 by DAS. Because of 100% mortality in the group not pretreated with DAS, plasma ALT values are unavailable for the 24-h time point in Fig. 9. These results (Figs. 8 and 9) collectively confirm that CYP2E1 plays a role in the bioactivation of TA in inflicting mechanism-based liver injury. Furthermore, these findings also suggest that highly increased mechanism-based TA liver injury in DR rats is due to DR-induced CYP2E1.

FMO Activity and Liver Injury Following I3C Administration. If FMO is involved in the bioactivation of TA, in vivo inhibition of FMO should result in decreased TA-induced liver injury. Dietary administration of I3C for 10 days decreased FMO1 activity in both AL and DR rats (Fig. 10). In AL rats hepatic FMO activity was reduced by 50% and in DR rats it was reduced by 33% (Fig. 10). However, TA-induced liver injury was substantially increased in the AL + I3C-treated rats at 24 h (Fig. 11) and in the DR + I3C-treated rats the change was not significant (Fig. 11). These results show that FMO inhibition by I3C does not result in decreased liver injury, suggesting that FMO is highly unlikely to be associated with increased TA liver injury. Indeed, liver injury was increased in AL rats with FMO inhibition, suggesting that hepatic FMO may be involved in the detoxification of TA. Because of lower FMO, a corresponding incremental amount of TA would become available for bioactivation by CYP2E1. It is possible that higher availability of TA for bioactivation by CYP2E1 may explain the higher liver injury.

Discussion
Earlier studies have shown that DR substantially increases mechanism-based liver injury of TA. Liver injury was >6-fold higher at a low dose (50 mg/kg i.p.) and >2.5-fold higher at lethal dose of TA (600 mg/kg i.p.) in DR rats compared with their AL cohorts (Ramaiah et al., 1998). Figure 6 represents liver injury of CCl₄ upon pretreatment with DAS. As expected, administration of CCl₄ (4 ml/kg i.p.) alone resulted in high liver injury as assessed by plasma ALT activity at 12 h. However, in the group pretreated with DAS, CCl₄ caused a 6-fold decrease in liver injury. Between 12 and 20 h after CCl₄ injection there was 100% mortality in the rats not receiving DAS pretreatment, while 50% survival occurred in the DAS-pretreated group (data not shown), confirming the in vivo inhibition of CYP2E1 by DAS. Because of 100% mortality in the group not pretreated with DAS, plasma ALT values are unavailable for the 24-h time point in Fig. 9. These results (Figs. 8 and 9) collectively confirm that CYP2E1 plays a role in the bioactivation of TA in inflicting mechanism-based liver injury. Furthermore, these findings also suggest that highly increased mechanism-based TA liver injury in DR rats is due to DR-induced CYP2E1.
Diet Restriction, CYP2E1, and Thioacetamide Liver Injury

Plasma ALT activity was determined at 12 and 24 h after the administration of TA. ALT was measured as a marker of liver injury after TA administration in the AL group. Results are expressed as mean ± S.E. (n = 4). * value significantly different from the AL group at the corresponding time point.

FIG. 9. Effect of diallyl sulfide (200 mg/kg i.p.) pretreatment on plasma ALT activity in ad libitum rats treated with a single dose of carbon tetrachloride (4 ml/kg).

FMO activity was measured according to Guo et al. (1992) by thiourea-dependent oxidation of thiocholine. Results are expressed as mean ± S.E.

FIG. 10. FMO activities in ad libitum and diet-restricted rats with and without 13C treatment after a low dose (50 mg/kg i.p.) of thioacetamide.

FIG. 11. Plasma ALT activities in ad libitum and diet-restricted rats with and without 13C treatment after a low dose (50 mg/kg i.p.) of thioacetamide.

ALT was measured as a marker of liver injury over a time course (0–24 h) after TA administration. Results are expressed as mean ± S.E.

FMO1, was not found to be involved in the bioactivation of TA (Wang et al., 2000b).

We hypothesized that CYP2E1 induced by DR is predominantly responsible for higher infliction of liver injury of TA in DR rats. Several lines of evidence support this hypothesis. CYP2E1 is present peripherally and TA is a strong perivenous toxicant (Ronis et al., 1996); DR is known to induce hepatic microsomal CYP2E1 (Leakey, 1989; Brown et al., 1995) and increased liver injury of hepatotoxins known to be bioactivated by CYP2E1 has been reported (Watkins et al., 1988; Cheli et al., 1990); and diabetes is known to be accompanied by induction of CYP2E1 (Watkins et al., 1988; Wang et al., 2000b) and diabetes is also known to increase liver injury of thioacetamide (El-Hawari and Plaa, 1983; Wang et al., 2000a). Additionally, participation of some of the other CYP450 isoforms can be ruled out based on literature reports (El-Hawari and Plaa, 1983; Qu et al., 1998). For instance, studies have shown that phenobarbital, 3-methylcholanthrene, and 3,4-benzo(a)pyrene were unable to potentiate TA liver injury, thereby ruling out other major CYP450 isoforms (CYP1A1/2 and CYP2B1/2) in the bioactivation of TA (El-Hawari and Plaa, 1983). Furthermore, recent studies have shown by immunoblotting techniques that CYP1A1, 1A2, 2B1, 4A1, and 4A3 are unchanged with fasting in male Fisher 344 rats (Qu et al., 1998), suggesting that these isoforms are unlikely to be involved in DR-induced enhancement of mechanism-based liver injury of TA in DR rats. However, the possibility of other remaining cytochrome P450 isoforms (CYP34, CYP2C, etc.) contributing to the bioactivation-based liver injury of TA remains to be investigated. Our data are consistent with such a possibility.

PYR and INZ (Kim et al., 1993; Park et al., 1993) are leading examples of relatively specific inducers of CYP2E1 and hence these compounds were used for in vivo induction of CYP2E1. A 2.5-fold increase in liver injury was observed in PYR + TA group compared with Veh + TA group. This corresponds very well with the 2.2-fold induction of CYP2E1 as revealed by Western blot analysis. Also, the peak of liver injury in PYR + TA group appeared at 24 h in contrast to 36 h observed in the Veh + TA group. A 3-fold increase in liver injury in PYR + TA group appeared at 24 h in contrast to 36 h observed in the Veh + TA group. A 3-fold increase in liver injury was observed in INZ + TA group compared with Veh + TA group. The same INZ pretreatment used in previous studies (Wang et al., 2000b), yielded a 2.5-fold induction of CYP2E1 protein as assessed by Western blot analysis. Liver injuries assessed by plasma ALT elevation were concordant with liver histopathology. Liver injury of TA as measured by ALT elevation corresponds well with the level of CYP2E1 induction by INZ. PYR and INZ pretreatments...
resulted in increased as well as earlier onset of TA liver injury. Highly increased liver injury of TA in DR rats is also accompanied by early onset of TA liver injury (Ramaiah et al., 1998a,b, 2001), observations consistent with the mediation by induced CYP2E1.

Chlorozoxazone 6-hydroxylation is a relatively specific probe for liver CYP2E1 catalytic activity (Bachmann and Sarver, 1996). Although contribution by other P450 isozymes is possible, this is currently being used both in vivo and in vitro studies as a measure of CYP2E1 activity in humans (Court et al., 1997). We investigated chlorozoxazone 6-hydroxylation in liver microsomal incubations derived from pyridine-pretreated DR and AL rats. Both total CYP450 content and CYP2E1 levels were increased by PYR pretreatment (Figs. 4, A and B, and 5), confirming induction of CYP2E1 isozyme by PYR. The 2-fold increase in total CYP450 content was accompanied by a 2.2-fold increase in CYP2E1 protein, and a corresponding 2-fold increase in CYP2E1 activity at 24 h following TA injection (Figs. 4, A and B, and 5). Since DR resulted in >6-fold increase in liver injury following 50 mg of TA/kg, total CYP450 content and CYP2E1 levels were quantified in DR and AL rats before and after TA administration. DR alone resulted in a significant 2-fold increase in total cytochrome 450 content (Fig. 6A) and a 4.6-fold increase in CYP2E1 protein (Fig. 7) and a 3-fold increase in CYP2E1 activity (Fig. 6B) suggesting that CYP2E1 is substantially induced by DR. Furthermore, 24 h following TA injection, there was an elevation in total cytochrome P450 content (2-fold) and CYP2E1 activity (2-fold) in AL rats, suggesting that TA itself is an inducer of CYP2E1. Induction of CYP2E1 by TA has not been reported before. However, no further increase in both total CYP450 content and CYP2E1 activity was evident in DR rats 24 h following TA injection (Fig. 6, A and B), indicating that TA could not induce CYP2E1 over and above the 4.6-fold elevation already induced by DR. This suggests that DR and TA share the same mechanism of CYP2E1 induction. DR is known to reduce serum insulin levels (Leaky et al., 1991a). Regarding the mechanism of CYP2E1 induction by DR, insulin is known to be decreased in DR (Leaky et al., 1991a, 1998) and insulin is known to suppress CYP2E1 by post-transcriptional mechanisms (Dong et al., 1988). Although we have not investigated whether CYP2E1 induction by DR and TA is exclusively by post-transcriptional mechanisms, it is likely that the mechanism of TA induction of CYP2E1 is by either stabilization of mRNA or CYP2E1 protein. However, at this time transcriptional mechanisms cannot be ruled out. In our studies, there was a good correlation of liver injury and CYP2E1 induction in both temporal manner and amount of induction. These findings support the role of CYP2E1 in the bioactivation of TA and DR-induced increase in CYP2E1 as the predominant basis of higher mechanism-based TA liver injury in DR rats.

To determine whether increased total CYP450 content and CYP2E1 activity in DR rats was merely due to decrease in liver weight, microsomal protein levels in AL and DR rats were normalized to liver weight (g). No significant differences were found in protein levels normalized to liver weight in AL and DR rats, even though there was a significant reduction (66%) in body weight gain and liver weight (52%, P ≤ 0.01) in DR rats compared with AL group. These results are consistent with the recent report on a series of induction studies where the authors have reported that the magnitude of the metabolizing enzyme induction is neither linked to hepatocyte size nor liver weight (Amacher et al., 1998).

DAS is a well known relatively selective irreversible in vivo inhibitor of CYP2E1, which inactivates CYP2E1 via a suicide-inhibitory action (Brady et al., 1991). DAS was used to inhibit hepatic microsomal CYP2E1 in the present in vivo studies. Recent studies have conclusively demonstrated with null mice lacking expression of CYP2E1 (CYP2E1<sup>-/-</sup>) that CYP2E1 is required for bioactivation of CCl<sub>4</sub> and consequent hepatotoxicity (Wong et al., 1998). Therefore, liver injury of CCl<sub>4</sub> can be used as an in vivo marker of CYP2E1 activity to ascertain inhibition of CYP2E1 by DAS. In vivo inhibition of CYP2E1 by DAS was confirmed by the marked decrease in CCl<sub>4</sub>-induced liver injury. Furthermore, this was also confirmed by 50% survival of rats receiving an ordinarily lethal dose of CCl<sub>4</sub>. If CYP2E1 is predominantly involved in the bioactivation of TA in the DR rats, pretreatment of DR rats with DAS should lead to a decrease in mechanism-based liver injury of TA. Liver injury of TA was substantially decreased (60%) when the DR rats were pretreated with DAS (Fig. 8). A clear correlation was found between CYP2E1 inhibition by DAS and decreased TA liver injury in these rats. Together, these results further support the view that CYP2E1 is involved in the liver injury of TA in normal rats and in the enhanced liver injury in DR rats.

FMO has been implied in the bioactivation of TA (De-Ferreyra et al., 1983). To test the involvement of FMOs in the metabolism of TA to its reactive metabolite, I3C, a relatively specific in vivo inhibitor of FMO was used. If FMO is involved in the bioactivation of TA, I3C-treated rats should experience lower liver injury due to inhibition of FMO activity. Both AL + I3C- and DR + I3C-treated rats had less FMO activity compared with the respective controls (Fig. 10). However, the liver injury of TA as measured by ALT elevations was increased in AL rats at 24 h and the increases at 12 and 24 h in the DR group (Fig. 11) were not statistically significant, despite significantly decreased FMO inhibition in both groups (Fig. 10). The mechanisms for increased TA liver injury in AL rats during FMO inhibition by I3C are unknown. It is plausible that in AL rats, FMO may be playing a detoxifying role in contrast to bioactivation. By blocking FMO detoxifying pathway, additional TA may become available for TA bioactivation by CYP2E1 causing increased liver injury as noted. Although less likely, I3C may induce other P450 isozymes such as CYP2E1, which is responsible for TA bioactivation. It is known that I3C induces a number of different CYP450 isoforms, including CYP1A, 2B, and 3A (Larsen-Su and Williams, 1996; Renwick et al., 1999). However, it is known that preexposure to 3-methylcholanthrene and/or phenobarbital, which induce CYP1A and CYP2B1, respectively, is not known to potentiate TA hepatotoxicity (El-Hawari and Plaa, 1983), indicating that neither CYP1A and CYP2B isoforms are likely to mediate TA bioactivation. Hence, induction of CYP450s by I3C is highly unlikely to play a role in enhanced TA liver injury in I3C-treated rats. Therefore, increased availability of TA for bioactivation via CYP2E1 is the likely mechanism for higher liver injury in rats with lower hepatic FMO.

In conclusion, the data presented in this study suggest involvement of CYP2E1 in increased TA liver injury in DR rats. Although FMO has been implicated in TA bioactivation, it is unlikely to be involved in the bioactivation of TA in AL rats and is also unlikely to contribute to highly exaggerated liver injury in DR rats. The results suggest a possible detoxication of TA by rat hepatic FMO.

Acknowledgments. We acknowledge Drs. Julian Leaky and John Seng, National Center for Toxicological Research (NCTR, Jefferson, AR) for help on CYP2E1 studies. We also acknowledge Dr. Daniel Ziegler (University of Texas, Austin, TX) for help on FMO studies. We are thankful to the Division for Toxicology of the American Society for Pharmacology and Experimental Therapeutics for an award to Shashi Ramaiah to present preliminary results of this article at the Experimental Biology Meeting (FASEB) held in Washington, DC, April 17–21, 1999.
References