THE EFFECT OF ISONIAZID ON CYP2E1- AND CYP4A-MEDIATED HYDROXYLATION OF ARACHIDONIC ACID IN THE RAT LIVER AND KIDNEY

Samuel M. Poloyac, Michael A. Tortorici, Danielle I. Przychodzin, Robert B. Reynolds, Wen Xie, Reginald F. Frye, and Michael A. Zemaitis

University of Pittsburgh School of Pharmacy, Department of Pharmaceutical Sciences, Pittsburgh, Pennsylvania (S.M.P., M.A.T., R.B.R., W.X., M.A.Z.); Shenandoah University, Bernard J. Dunn School of Pharmacy, Winchester, Virginia (D.I.P.); and University of Florida College of Pharmacy, Department of Pharmacy Practice, Gainesville, Florida (R.F.F.)

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ABSTRACT:

Cytochrome P450 (P450) bioactivation of arachidonic acid to hydroxyeicosatetraenoic acids (HETEs) has been reported to be isoform- and tissue-specific. To determine whether altered P450 expression affects the production of these metabolites, the formation of HETEs after isoniazid-mediated CYP2E1 induction was evaluated in the rat liver and kidney. Male Sprague-Dawley rats received isoniazid (200 mg/kg) or saline intraperitoneally once daily for 5 days. Chlorozoxazone, lauric acid, and arachidonic acid hydroxylation was measured in liver and kidney microsomes with and without preincubation with the specific CYP2E1 inhibitor, trans-1,2-dichloroethylene (DCE). P450 isoform content and tissue HETE metabolite concentrations were also determined. Isoniazid increased CYP2E1 protein, and the 6-hydroxychlorzoxazone formation rate was increased by 2.7 ± 0.3- and 2.2 ± 0.5-fold in liver and kidney, respectively. Formation of 19-HETE and 11-hydroxylauric acid was induced 2.3 ± 0.6-fold and 2.2 ± 0.4-fold in the liver, respectively, with no difference in the kidney. All of the induced activities were attenuated by DCE. An unanticipated decrease in liver CYP4A expression and in vitro 20-HETE formation rate was observed after isoniazid administration. Isoniazid decreased liver and kidney 20-HETE content to 34 ± 10% and 15.6 ± 5.3% of control, respectively, without significantly altering tissue 19-HETE concentration. Based on these findings, we conclude that under induced conditions, CYP2E1 is a primary enzyme involved in liver, but not kidney, formation of 19-HETE. In addition, formation of both CYP4A and 20-HETE is reduced in the liver by isoniazid. It was also demonstrated that tissue concentrations parallel in vitro inhibited formation rates for 20-HETE, but not the induced 19-HETE formation in the liver.

The cytochrome P450 (P450) enzyme superfamily metabolizes a wide array of exogenous and endogenous substrates. The importance of P450-mediated metabolism of xenobiotics is well known. P450 enzymes also have a less appreciated, but equally important role in the metabolism of endogenous substrates to bioactive intermediates involved in a wide variety of physiologic processes. One such P450-mediated bioactivation pathway is the monohydroxylation of arachidonic acid to produce hydroxyeicosatetraenoic acids (HETEs) and epoxidation to form epoxygenosatrienoic acids (Capdevila et al., 1981; Schwartzman et al., 1985). Of the multitude of HETE metabolites that have been identified, 19-HETE, 11-HETE, and 15-HETE are the most abundant and have been implicated in a variety of physiologic processes (Wang et al., 1994; Gebremedhin et al., 2000), and mesenteric arteries (Wang et al., 2001), which is mediated by decreasing calcium-dependent large-conductance potassium channel influx and increasing L-type calcium channel influx into vascular smooth muscle (Zou et al., 1996; Gebremedhin et al., 1998). Recently, 20-HETE has also been implicated as a second messenger that mediates vascular endothelial growth factor-dependent skeletal muscle angiogenesis after electrical stimulation (Amaral et al., 2003). Collectively, these data suggest that the regiospecific hydroxylation of arachidonic acid by specific cytochrome P450 isoforms may act as a mechanism to regulate the production of HETE metabolites and their associated physiologic effects.

Consistent with the theory of differential P450 isoform regulation

ABBREVIATIONS: P450, cytochrome P450; 6OH-CZN, 6-hydroxychlorzoxazone; HETE, hydroxyeicosatetraenoic acid; DCE, trans-1,2-dichloroethylene; INH, isoniazid hydrazide.

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altering HETE metabolite production are studies demonstrating that 19-HETE and 20-HETE are formed by different P450 isoforms. Both in vitro expression studies and inhibition studies have implicated isoforms of the CYP4 family in the production of 20-HETE. In the rat, CYP4A1, 4A2, 4A3, 4A8, 4F1, and 4F4 have all been shown to catalyze the formation of 20-HETE (Sundseth and Waxman, 1992; Nguyen et al., 1999; Xu et al., 2004). The formation of 19-HETE has been attributed to CYP2E1 (Laethem et al., 1993) and CYP4A isoforms (Nguyen et al., 1999) in the rat and CYP2C (Luo et al., 1998) and CYP2J9 (Qu et al., 2001) isoforms in the mouse. Interestingly, the involvement of individual P450 isoforms in the formation of these metabolites appears to be tissue-specific and dependent on the level of expression of a given P450 isoform within a given tissue. This tissue specificity has been most evident in studies that evaluated the role of CYP2E1 induction on the formation of HETE metabolites. In separate studies, Amet et al. (1994) showed that under induced conditions, CYP2E1 is responsible for the formation of 19-HETE in the liver (Amet et al., 1994), but not in the kidney (Amet et al., 1997). Although these studies did demonstrate the induction of 19-HETE formation with known CYP2E1 inducers, a study that systematically evaluates tissue-specific induction and inhibition at saturating concentrations of arachidonic acid has not been conducted. In addition, other fatty acids are known to undergo ω- and ω-1-hydroxylation by P450 enzymes. Most notable is the metabolism of lauric acid to its 11-hydroxy- and 12-hydroxylauric acid metabolites, which are used as index reactions to characterize CYP4A isoform activity in the rat (Hardwick et al., 1987; Hoch et al., 2000). Based on these previous data, it was the purpose of this study to determine the role of CYP2E1 in the hydroxylation of arachidonic acid and lauric acid in both the rat liver and kidney after CYP2E1 induction by isoniazid (Park et al., 1993). The effect of trans-1,2-dichloroethylene (DCE), a specific CYP2E1 inhibitor (Mathews et al., 1998), on CYP2E1-mediated fatty acid metabolism was also evaluated.

Materials and Methods

Animals. Arachidonic acid, 20-HETE, and [3H]15(S)-HETE metabolites were purchased from Cayman Chemical (Ann Arbor, MI). 12-Hydroxylauric acid was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). Chloroxazone and 6-OH-chloroxazone were purchased from Sigma-Aldrich (St. Louis, MO). P450 antibodies were purchased from BD Gentest (Woburn, MA). Organic solvents were ordered from Fisher Scientific Co. (Pittsburgh, PA) and all other chemicals were ordered from Sigma-Aldrich unless otherwise specified.

Animals. Male Sprague-Dawley rats (200–225 g; Hilltop Laboratory Animals, Inc., Scottsdale, PA) were maintained in cages and fed a diet of pellets and water ad libitum. Rats received intraperitoneal (i.p.) injections of isoniazid in saline (isonicotinic acid hydrazide; Sigma-Aldrich) 200 mg/kg once daily for 5 days. Control animals were injected i.p. with sterile normal saline. Animals were sacrificed 3 h after the last injection, and liver and kidney tissue was excised. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Microsome Preparation. The liver and kidney tissues were collected and homogenized with microsomes were prepared as previously described by Rockich and Blouin (1999), via differential centrifugation in a Beckman L8-70 ultracentrifuge (Beckman Coulter, Fullerton, CA). Total microsomal protein was determined and the quality of harvested liver microsomes was determined by spectral P450 analysis as described by Omura and Sato (1964).

Assay of CYP2E1 Activity. Microsomal incubations contained 400 μg of microsomal total protein, 400 μM chloroxazone, and 1 mM NADPH in a final volume of 1 ml and were incubated at 37°C for 20 min. The reaction was stopped with 50 μl of 42.5% o-phosphoric acid followed by the addition of the internal standard, umbelliferone. Samples and standards were then extracted in...
5 ml of ethyl acetate, dried under nitrogen gas, and reconstituted in 200 μl of mobile phase. Detection of CYP2E1-dependent 6OH-CZN activity was determined based on previously described methods (Jayyosi et al., 1995) with modifications for detection via mass spectrometry. Briefly, compounds were separated with a Waters 2690 Separations Module (Waters, Milford, MA) using a Symmetry C8 column (5 μm, 2.1 × 150 mm). The mobile phases consisted of double distilled H2O and methanol (60:40 gradient to 0:100 over 10,000 g). Extracted twice with 3 ml of diethyl ether. Free liver tissue concentrations (chlorzoxazone), 184.1 m, 2.1 °p/H11021 0.01. Measurements were performed with Xcalibur software version 1.2 (Thermo Finnigan). The ratio of HETE metabolites to internal standard areas was determined and metabolite content was quantified from the linear standard curve for 20-HETE and 12-hydroxyauric acid standards. Percentage of control values for 19-HETE and 11-hydroxyauric acid were calculated from the metabolite area to internal standard ratio.

**DCE Inhibition Study.** Microsomes from saline-treated or isoniazid-treated animals (n = 6 per treatment group) were preincubated with DCE or vehicle as described by Mathews et al. (1998). Incubations containing 3000 μg of microsomal protein in 2 ml of buffer with and without DCE were initiated by the addition of 1 mM NADPH, and incubations were carried out for 30 min at 37°C. Reactions were stopped by placing the sample tubes on ice. DCE- and vehicle-treated microsomes were re-isolated by centrifugation for 30 min at 100,000 g with a Beckman L8-70 ultracentrifuge (Beckman Coulter). Resolated microsomes were pooled to yield three pools of microsomes per treatment group. Activity analysis using chloroauric acid and arachidonic acid were completed as described above.

**Western Blot Analysis.** A 10% SDS-polyacrylamide gel was used to separate the microsomal proteins by electrophoresis. The gel was run at 200 V for 45 min. The proteins were transferred to nitrocellulose and blocked with 5% milk. The nitrocellulose was probed with goat anti-rat CYP2E1 or anti-rat CYP4A polyclonal antibody from BD Gentest. After washing, an alkaline phosphatase conjugate monoclonal anti-goat IgG was applied followed by CDP-Star with Nitroblock II substrate (Tropix, Bedford, MA) for enhanced chemiluminescence. Appropriate washing was completed between all blocking and antibody steps.

**Statistical Analysis.** Statistical significance was determined via a two-sample unpaired t test assuming equal variance. For the inhibition study, an analysis of variance with a Bonferroni correction was carried out assuming equal variance. The alpha level for significance was set in advance at p < 0.05.

**Results**

**Effect of Isoniazid on CYP2E1 Protein and Enzymatic Function in Rat Liver and Kidney.** Treatment of rats with isoniazid intraperitoneally daily for 5 days resulted in anticipated induction in CYP2E1 protein content and functional activity as assessed by 6-hydroxylation of chloroauric acid in both the rat kidney and liver (Fig. 1). Comparable levels of induction of 6OH-CZN formation were observed in kidney and liver microsomes with 2.2 ± 0.5-fold induction observed in kidney microsomes and 2.7 ± 0.3-fold induction of 6OH-CZN formation in liver microsomes from isoniazid-treated animals. The induction in functional activity was
The results of this research demonstrate that the inducibility of ω-1-hydroxylation of arachidonic and lauric acid is tissue-specific in isoniazid-treated animals. In addition, the divergent effects of isoniazid on ω- versus ω-1-hydroxylation of fatty acids in the liver demonstrate that differential regulation of P450 isoforms produces paralleled by an increase in CYP2E1 immunoreactivity in the kidney and liver of isoniazid-treated animals. In addition, the level of 6OH-CZN formation in the kidney microsomes was approximately 3 to 4% of liver formation rate, which is consistent with previous reports (Poloyac et al., 2000).

**Effect of Isoniazid on Arachidonic Acid Hydroxylation in Rat Liver and Kidney.** Induction of CYP2E1 by isoniazid exhibited clear tissue-specific effects on arachidonic acid hydroxylation. In the liver, the formation of 19-HETE was significantly increased 2.3 ± 0.6-fold in isoniazid-treated animals (Fig. 2A). In sharp contrast, no significant difference in 19-HETE formation was observed in kidney microsomes obtained from control and isoniazid-treated animals. In contrast to the observations with 19-HETE, the formation of 20-HETE was significantly reduced in both rat liver and kidney microsomes from isoniazid-treated animals (Fig. 2B). Control 20-HETE formation rates were 0.564 ± 0.184 nmol/mg/min and 0.910 ± 0.206 nmol/mg/min for kidney and liver, respectively.

**Effect of Isoniazid on Lauric Acid Hydroxylation in Rat Liver and Kidney.** The formation of 11-hydroxylic acid was increased 2.2 ± 0.4-fold in the liver, but not in the kidney (Fig. 3A). This observed pattern of induction was similar to that observed for 19-HETE formation from arachidonic acid. In addition, the formation of 12-hydroxylic acid paralleled the results observed with terminal hydroxylation of arachidonic acid by demonstrating a significant reduction to 48 ± 12% of control values in the liver microsomes from isoniazid-treated animals (Fig. 3B). The observed reduction in liver 12-OH-lauric acid formation and 20-HETE formation was paralleled by a reduction in the liver CYP4A immunoreactivity in isoniazid-treated animals as compared with control animals (Fig. 3D). A trend toward a reduction in CYP4A immunoreactivity was observed in the lower band from kidney microsomes with no apparent effect on the upper band’s immunoreactivity (Fig. 3C).

**Effect of DCE Preincubation on Hydroxylation of Chlorzoxazone and Arachidonic Acid in Microsomes from Isoniazid-Induced Animals.** Microsomes were incubated with DCE and subsequently re-isolated by centrifugation. This method of microsomal incubation with DCE was used to provide maximal selectivity of inhibition for the CYP2E1 isoform as demonstrated by Mathews et al. (1998). Preincubation with DCE reversed the INH-induced formation of 6-hydroxylauric acid in kidney and liver microsomes by 65 ± 18% and 70 ± 4%, respectively, in pooled DCE-pretreated microsomes (Fig. 4A). In addition, DCE inhibited formation of 19-HETE by 73 ± 22% in isoniazid-induced liver microsomes, whereas no significant alteration was observed in kidney microsomes (Fig. 4B). The formation rate of 20-HETE was not altered by DCE pretreatment (data not shown).

**Effect of Isoniazid on Free, Endogenous 19-HETE and 20-HETE Tissue Concentrations.** Based on the observed alterations in the microsomal formation of HETE metabolites in isoniazid-treated animals, liver and kidney tissue was extracted for determination of free endogenous HETE metabolite content. Liver tissue from isoniazid-treated animals demonstrated no significant alteration in the free 19-HETE content as estimated by the 19-HETE content to internal standard ratio normalized to gram tissue weight (Fig. 5A). Similar evaluation of 20-HETE content demonstrated a significant reduction to 34 ± 10% of control levels (Fig. 5B) in the liver extracts. The kidney demonstrated similar alterations with no significant alteration in the 19-HETE (Fig. 5A) content and a reduction in 20-HETE content to 15.6 ± 5.3% of control animals (Fig. 5B).

**Discussion**

The results of this research demonstrate that the inducibility of ω-1-hydroxylation of arachidonic and lauric acid is tissue-specific in isoniazid-treated animals. In addition, the divergent effects of isoniazid on ω- versus ω-1-hydroxylation of fatty acids in the liver demonstrate that differential regulation of P450 isoforms produces
CYP2E1 and 3A4 (Sinclair et al., 1998). The data in the present study
Robottom-Ferreira et al., 2003). Ethanol has been shown to induce
induce 2C, 2E, and 2J, along with other isoforms (Park et al., 1993;
Park et al., 1993), as well as CYP2E1. Pyrazole has been reported to
pyridine has been shown to induce 1A1 (Iba et al., 2002) and 2B1/2B2
forms, including 1A, 2B, and 2E (Chen and Ueng, 1997). Similarly,
isoforms. Acetone has been reported to induce multiple P450 iso-
many of the inducers used have been shown to induce multiple P450
alterations in the production of hydroxylated fatty acid metabolites
in vivo and in vitro. This study also demonstrated that in vivo liver
tissue concentrations of 20-HETE parallel in vitro reductions in
microsomal formation rate, whereas inducibility of 19-HETE ob-
served under saturable incubation conditions was not paralleled by
in vivo free tissue concentrations.

Different P450 isoforms have been shown to favor production of
certain monohydroxylated products, specifically, ω- or ω-1-hydroxy-
lation. Consistent with the results in the present study are several
studies suggesting a role for CYP2E1 in fatty acid hydroxylation.
Laethem et al. (1993) reported an increase in 19-HETE formation in
the liver after induction of CYP2E1 by acetone exposure. Amet et al.
(1994) reported that induction of CYP2E1 with either pyridine, pyra-
zole, ethanol, or acetone produced an increase in 11-OH (ω-1-hy-
droxylation) lauric acid formation in the liver. Although these studies
suggest a role for CYP2E1 in the ω-1-hydroxylation of fatty acids,
many of the inducers used have been shown to induce multiple P450
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forms, including 1A, 2B, and 2E (Chen and Ueng, 1997). Similarly,
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Robottom-Ferreira et al., 2003). Ethanol has been shown to induce
CYP2E1 and 3A4 (Sinclair et al., 1998). The data in the present study
provide additional evidence for the role of CYP2E1 in the formation
of 19-HETE in the liver, based on the specific induction of CYP2E1
by isoniazid coupled with the results of DCE inhibition.

To confirm the role of CYP2E1 in the isoniazid-induced formation
of 19-HETE, microsomal inhibitions were conducted using DCE. Previous studies demonstrated that DCE is a specific CYP2E1 mecha-
anism-based inhibitor (Lilly et al., 1998). The metabolism of DCE is
required for interaction with the P450 heme type II binding site (Costa
and Ivanetich, 1982). In addition, DCE is thought to competitively
inhibit other P450 isoforms; however, the nonspecific inhibition is
avoided by preincubation and re-isolation of microsomal fractions as
demonstrated by Mathews et al. (1998). In the study by Mathews et al.
(1998), the selectivity of this inhibition was demonstrated on probe
substrates for CYP2E1, 1A2, 2A1, 2C6, 2C11, 2D1, and 3A
isoforms, with significant inhibition occurring with only CYP2E1-
mediated hydroxylation of p-nitrophenol (>80% inhibition as com-
pared with control). The present study demonstrated significant
inhibition of 6-hydroxychlorzoxazone and 19-HETE formation without
significant alterations in 20-HETE formation after re-isolation of rat
liver microsomes preincubated with DCE. The in vitro attenuation of
the induced 6OH-CZN and 19-HETE formation rates in microsomes
from isoniazid-treated animals by DCE strongly suggests that induced
CYP2E1 is responsible for the hepatic induction of 19-HETE forma-
tion with isoniazid exposure.

The observations in kidney microsomes in the present study are
consistent with the results from Amet et al. (1997), who reported that fatty acid ω-1-hydroxylation (arachidonic acid and lauric acid) was not altered by CYP2E1 induction in the kidney. In the study by Amet et al. (1997), arachidonic acid metabolism was measured by incubation with nonsaturating concentrations of 15-C-arylcholine substrate. In the current study, an liquid chromatography-mass spectrometry method was used to assess enzymatic formation rates at saturable concentration; therefore, enzymatic formation was dictated by alterations in P450 enzyme levels in the tissues evaluated. The most probable cause for the lack of induction of 19-HETE formation in the rat kidney is the low basal expression levels of CYP2E1 in the kidney. CYP2E1 activity has been reported to be <10% of liver activity. Even after ~2-fold induction of CYP2E1 protein and functional activity in kidney microsomes, the levels of this isozyme are relatively low as compared with total kidney P450 content. It is likely that other P450s present in the kidney at higher levels are primarily responsible for the formation of 19-HETE in the rat kidney. It is also possible that the increase in CYP2E1 in the kidney, coupled with a reduction in the CYP4A isoforms in the kidney, resulted in no significant change in 19-HETE; however, further studies are necessary to determine the effect of specific isoforms in kidney microsomes.

An unanticipated reduction in CYP4A expression and ω-hydroxylation of both lauric and arachidonic acid was observed in rat liver. A previous study by Okita et al. (1997) demonstrated that CYP4A2 is the predominant Western blot band in Sprague-Dawley liver and kidney microsomes using the same antibody used in these studies. It is likely that the lower bands in both the liver and kidney blots represent the CYP4A2 isoform. It is important to note that the alterations in fatty acid metabolism may not be only due to alterations in CYP4A, but also may be due to other P450 isoforms such as CYP4F isoforms.

The observed alteration in the in vitro formation rate of 20-HETE was paralleled by decreased free tissue 20-HETE concentration in isoniazid-treated animals, whereas the free liver 19-HETE concentration was not different despite induced microsomal formation of 19-HETE in the kidneys of 19-HETE-treated animals, whereas the free liver 19-HETE concentration was paralleled by decreased free tissue 20-HETE concentration in the kidneys of 19-HETE-treated animals. Further studies are necessary to determine the mechanism(s) by which alterations in fatty acid metabolism may not be only due to alterations in CYP4A, but also may be due to other P450 isoforms such as CYP4F isoforms.

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Address correspondence to: Dr. Samuel M. Poloyac, Assistant Professor, 808A Salk Hall, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA 15261. E-mail: poloyac@pitt.edu