In Vivo Mechanistic Studies on the Metabolic Activation of 2-Phenylpropionic Acid in Rat

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ABSTRACT
Two alternative metabolic pathways, acyl glucuronidation and acyl-CoA formation, are implicated in the generation of reactive acylating metabolites of carboxylic acids. Here, we describe studies that determine the relative importance of these two pathways in the metabolic activation of a model substrate, 2-phenylpropionic acid (2-PPA), in vivo in rats. Male Sprague-Dawley rats were pretreated with and without (−)-borneol (320 mg/kg i.p.), an inhibitor of acyl glucuronidation, or trimethylacetic acid (TMA, 500 mg/kg i.p.), an inhibitor of acyl-CoA formation, before receiving 2-PPA (racemic, 130 mg/kg). After administration of 2-PPA, livers were collected over a 2-h period and analyzed for 2-PPA acyl glucuronidation and 2-PPA-CoA formation by high-performance liquid chromatography. Covalent binding was measured by scintillation counting of washed liver protein precipitates. Results showed that pretreatment with TMA led to a 49% decrease in covalent binding of 2-PPA to liver proteins, when a 64% decrease in the exposure of 2-PPA-CoA was observed. Conversely, 95% inhibition of acyl glucuronidation by (−)-borneol, led to a 23% decrease in covalent binding to protein. These results suggest that metabolic activation by 2-PPA-CoA formation contributes to covalent adduct formation to protein in vivo to a greater extent than metabolic activation by acyl glucuronidation for this model substrate.

2-Arylpropionic acids (profens) constitute a widely used class of nonsteroidal anti-inflammatory drugs that have been associated with a rare, but sometimes severe, idiosyncratic hepatotoxicity (Zimmerman, 1994; Boelsterli et al., 1995). The mechanisms underlying profen-induced liver toxicity are poorly understood, although reactive metabolites of profen drugs are often believed to mediate the idiosyncratic toxicity by binding covalently to proteins (Boelsterli et al., 1995; Pumford and Halmes, 1997).

Two alternative metabolic pathways (Fig. 1), acyl glucuronidation and acyl-CoA formation, have been suggested to generate reactive acylating metabolites of profen drugs (Boelsterli, 2002; Grillo and Benet, 2002). Acyl glucuronidation is a major route for the biotransformation and elimination of profen drugs, such as ibuprofen, carprofen, ketoprofen, naproxen, and fenoprofen (Spahn-Langguth et al., 1997; Li and Benet, 2002). It is well established that acyl glucuronides are chemically reactive electrophiles that readily undergo nucleophilic displacement reactions with 1) water or hydroxyl anions in buffer, resulting in hydrolysis of the acyl-linked ester bond of acyl glucuronides to provide the aglycone; 2) hydroxyl groups on the glucuronic acid ring, resulting in intramolecular acyl migration to yield β-glucuronidase-resistant isomers (Spahn-Langguth and Benet, 1992; Hayball, 1995; Li and Benet, 2002); and 3) glutathione (GSH), resulting in the formation of S-acyl glutathione conjugates (Shore et al., 1995; Grillo and Benet, 2002; Li et al., 2002b; Olsen et al., 2002). More importantly, these acyl-linked glucuronides readily react with protein nucleophiles, leading to covalent binding of profen drugs to proteins both in vitro and in vivo (Spahn-Langguth and Benet, 1992; Hayball, 1995; Li and Benet, 2002).

Acyl-CoA formation is the key step for the unidirectional chiral inversion of profen drugs from the pharmacological inactive (R)- to the active (S)-enantiomer (Nakamura et al., 1981; Caldwell et al., 1988; Hall and Quan, 1994). The activated acyl-CoA derivatives of profen drugs also serve as obligatory intermediates for the formation of amino acid conjugates (Hutt and Caldwell, 1990), acyl-carnitine and acylcholine derivatives (Sastry et al., 1997), as well as hybrid triglycerides (Fears, 1985; Williams et al., 1986; Sallustio et al., 1988). All these processes require a reactive thioester

ABBREVIATIONS: GSH, glutathione; 2-PPA, 2-phenylpropionic acid; 2-PPA-1-O-G, 2-PPA-1-acyl glucuronides; 2-PPA-CoA, 2-PPA-S-acyl CoA; TMA, trimethylacetic acid; TMA-CoA, TMA-S-acyl-CoA thioester; HPLC, high-performance liquid chromatography; ACN, acetonitrile; CNS, central nervous system; AUC, area under the curve.
bond, and thus acyl-CoA derivatives are believed to be electrophilic in nature. Sallustio et al. (2000) demonstrated that covalent binding of nafenopin to human liver proteins is directly associated with formation of a nafenopin acyl-CoA thioester intermediate. A number of studies on protein fatty acylation have shown that endogenous acyl-CoA thioesters, including palmitoyl-CoA and arachidonoyl-CoA, can react nonenzymatically with sulfhydryl groups on proteins and peptides in vitro in a time- and concentration-dependent manner (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). Our recent studies demonstrate that the acyl-CoA thioester derivative of 2-phenylpropionic acid (2-PPA) is able to acylate GSH sulfhydryl to form the 2-PPA-S-acyl glutathione (2-PPA-SG) at a rate that was approximately 70-fold more rapid than the similar reactions with 2-PPA-1-O-acyl glucuronides (2-PPA-1-O-G) (Li et al., 2002b). 2-PPA-S-acyl-CoA (2-PPA-CoA) was also shown to be able to bind covalently to proteins, such as bovine serum albumin (Li et al., 2002b) and hepatic proteins (Li et al., 2002a).

Most profen drugs are generally metabolized in vivo to acyl glucuronides and acyl-CoA thioesters, both of which have been demonstrated to be chemically reactive and believed to mediate, at least in part, covalent adduct formation in vivo. The present studies were designed to examine the contribution of 2-PPA acyl glucuronidation and 2-PPA-CoA formation to 2-PPA-protein covalent adduct formation in vivo in rat liver. The results reported here strongly suggest that metabolic activation of 2-PPA by 2-PPA-CoA formation contributes to covalent adduct formation to protein in vivo to a greater extent than metabolic activation by 2-PPA acyl glucuronidation.

Materials and Methods

Materials. (R,S)-2-PPA, perchloric acid (70%), trimethylacetic acid (TMA), and [(1S)-endo]-(-)-borneol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diethyl ether was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Corn oil and trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Hionic-Fluor scintillation fluid was purchased from PerkinElmer Life Sciences (Boston, MA). (R,S)-[1-14C]2-PPA was synthesized by American Radiolabeled Chemicals (St. Louis, MO). Synthetic 2-PPA-CoA and the biosynthetic 2-PPA-1-O-G were available from previous studies in this laboratory (Li et al., 2002b). TMA-S-acyl-CoA (TMA-CoA) was synthesized by conventional procedures using chloroform, as we reported previously (Li et al., 2002b). All solvents used for HPLC analysis were of chromatographic grade.

Animals. Male Sprague-Dawley rats (250–300 g) were purchased from Bantan and Kingman Universal (Livermore, CA) and maintained in a controlled housing environment with 12-h light/dark cycles and received standard laboratory chow and water ad libitum. Rats were acclimated (for at least 3 days) to the housing conditions before use in experiments. All animal use was approved by the University of California San Francisco Committee on Animal Research.

In Vivo Inhibition Studies with 2-PPA. To determine the optimal inhibitory doses of (–)-borneol and TMA, dose-dependent inhibitory effects on 2-PPA acyl glucuronidation and 2-PPA-CoA formation were examined in vivo in rats. Briefly, rats received one of the following i.p. pretreatment regimens: 1) 0.9% saline (1 ml/rat, 10 min); 2) corn oil (1 ml/rat, 10 and 30 min); 3) (–)-borneol (160–480 mg/kg in corn oil, 1 ml/rat, 30 min); 4) TMA (400–800 mg/kg in corn oil, 1 ml/rat, 10 min), before receiving 2-PPA (130 mg/kg in 0.9% saline i.p., 0.5 ml/rat). Rats were then sacrificed 2 h after 2-PPA administration, and their livers were removed and immediately frozen in liquid nitrogen. In these preliminary studies, one rat was used for each dosage treatment. Pretreatment of TMA 10 min before 2-PPA dosing had approximately the same inhibitory effect on the metabolic activation of 2-PPA as the pretreatment 30 min before 2-PPA dosing. Pretreatment of TMA 30 min before 2-PPA dosing was used in later studies.

Analysis of 2-PPA Acyl Glucuronides in Livers of 2-PPA-Treated Rats. Analysis of 2-PPA-CoA in Livers of 2-PPA-Treated Rats. 2-PPA-CoA was extracted from the liver of 2-PPA-treated rats using a modification of the method described previously for extraction of acid-soluble acyl-CoA (Bhuiyan et al., 1988). Briefly, frozen rat liver (1.0 g) was homogenized in 1.5 ml of potassium phosphate buffer (0.05 M, pH 5) on ice. The resultant liver homogenate was immediately denatured by 0.75 ml of HClO4 (7%), mixed vigorously, and centrifuged (10,000 rpm). The supernatant was immediately denatured by the addition of 0.5 ml of acetonitrile (ACN). After centrifugation at 10,000g for 10 min, the supernatant was analyzed by reverse-phase HPLC for the formation of 2-PPA-CoA thioester. Protein pellets from the liver of (R,S)-[1-14C]2-PPA-treated rats were used to determine the covalent adduct formation.

Analysis of 2-PPA Acyl Glucuronides in Livers of 2-PPA-Treated Rats. For the analysis of 2-PPA acyl glucuronidation in rat liver, frozen rat liver (0.5 g) was homogenized in 0.5 ml of potassium phosphate buffer (0.05 M, pH 5) on ice. The resultant liver homogenate was immediately denatured by the addition of 0.5 ml of acetonitrile (ACN). After centrifugation at 10,000g for 10 min, the supernatant was analyzed by HPLC for determination of 2-PPA acyl glucuronides.

Covalent Binding of (R,S)-[1-14C]2-PPA to Rat Liver Proteins. Covalent binding of 2-PPA to proteins was measured by scintillation counting of exhaustively washed hepatic protein precipitates as we described previously (Li et al., 2002a) with minor modifications. Briefly, protein pellets from the livers of (R,S)-[1-14C]2-PPA-treated rats were washed seven times with 6 ml of a
solution of 0.05 M potassium phosphate buffer (pH 4.5) and 7% perchloric acid (3:1, v/v), mixed vigorously for 5 min, and centrifuged (1500g, 5 min) until no radioactivity was detected by scintillation counting in the resultant supernatants. The washing process was continued using a solution of methanol and ethyl ether (3:1, v/v, 7 × 6 ml), followed by another washing process with a solution of 80% methanol (7 × 6 ml). No radioactivity was detected in the supernatants of the final wash. After the final supernatants were removed, the washed pellets were left to dry at room temperature. The dry pellets were dissolved in 1 N NaOH (1.5 ml) at 80°C overnight. The pellets were dissolved in 1 N NaOH (1.5 ml) at 80°C overnight. The washes were left to dry at room temperature. The dry pellets were dissolved in 1 N NaOH (1.5 ml) at 80°C overnight. The washed pellets were left to dry at room temperature. The dry pellets were dissolved in 1 N NaOH (1.5 ml) at 80°C overnight. The 2-PPA acyl glucuronides in rat livers were performed by reverse-phase HPLC of perchloric acid extracts with isocratic elution and UV-detection at 262 nm (absorbance maximum for CoA; Fig. 2).

**HPLC Analysis.** Determination of 2-PPA-CoA formation and acyl glucuronidation in rat livers was carried out by reverse-phase HPLC, as we described previously (Li et al., 2002a). Briefly, HPLC analysis was carried out on a gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadzu, Kyoto, Japan) with an SCL-10A controller and an SPD-10A UV-Vis detector (both from Shimadzu). The 2-PPA-CoA in rat liver was analyzed on a SB-C8 Zorbax column (150 × 4.6 mm; Varian Analytical Instruments, Walnut Creek, CA) at a flow rate of 1.0 ml/min. The isocratic mobile phase containing 17.5% ACN in 0.19 M ammonium acetate buffer (pH 7.0) was used with UV detection at 262 nm. The 2-PPA acyl glucuronides in the liver were quantified by reverse-phase HPLC using an isocratic elution with 0.1% trifluoroacetic acid and 15% ACN on a Microsorb-MV C18 column (150 × 4.6 mm, 5 μm; Varian Analytical Instruments, Walnut Creek, CA) at a flow rate of 1.8 ml/min and detected by UV absorbance (226 nm). The identity of these metabolites was confirmed by mass spectrometry, which was described previously (Li et al., 2002a). Quantitative measurements of 2-PPA-CoA and 2-PPA-acyl glucuronide formation were made using a standard curve generated from absolute peak areas, by spiking liver samples of untreated rats with synthetic 2-PPA-CoA or biosynthetic 2-PPA-1-O-G, followed by processing as described above.

**Statistical Analysis.** Analysis of variance analysis of the 2-h studies with radiolabeled compound indicated statistically significant differences for mean values in treatment groups for all comparisons: covalent binding, 2-PPA-CoA and 2-PPA glucuronide. Pairwise multiple comparisons were analyzed using the Student-Newman-Keuls method with significance set at \( p < 0.05 \).

**Results**

**HPLC Analysis.** The analysis of 2-PPA-CoA formed in rat liver was performed by reverse-phase HPLC of perchloric acid extracts with isocratic elution and UV-detection at 262 nm (absorbance maximum for CoA; Fig. 2). Results showed that 2-PPA-treated rats form 2-PPA-CoA thioester (retention time of 6.3 min) in liver (Fig. 2A), which coeluted with synthetic standard 2-PPA-CoA (data not shown). HPLC analysis of liver extract from saline-treated rats showed no peak eluting at the retention time of 2-PPA-CoA (data not shown). Pretreatment with (-)-borneol had little effect on 2-PPA-CoA formation (Fig. 2, A and B), whereas 2-PPA-CoA markedly decreased when rats were pretreated with TMA (Fig. 2C). TMA-CoA was also detected in TMA-pretreated rats with retention time of 4.3 min (Fig. 2C), which coeluted with synthetic standard TMA-CoA (data not shown).

The analysis of 2-PPA acyl glucuronides formed in livers from 2-PPA-treated rats was also performed by reverse-phase isocratic HPLC (Fig. 3), which allowed for the separation of (R)- and (S)-2-PPA-1-O-G, eluting at 11.0 and 12.0 min, respectively, as well as 2-PPA acyl glucuronide migration isomers (data not shown). No peaks with the same HPLC retention times as the 2-PPA acyl glucuronides were observed during the HPLC analysis of liver extract from saline-treated rats (data not shown). Pretreatment of TMA decreased the total formation of 2-PPA acyl glucuronides. The \( R/S \) ratio of 2-PPA acyl glucuronides was increased in TMA-pretreated rats presumably by selectively increasing (R)-2-PPA-1-O-G levels and decreasing (S)-2-PPA-1-O-G levels (Fig. 3C), which is consistent with the inhibitory effect of TMA on 2-PPA-CoA formation. 2-PPA-CoA formation is a key step for chiral inversion of 2-PPA from (R)- to (S)-isomers. Pretreatment with (-)-borneol significantly decreased 2-PPA acyl glucuronidation (Fig. 3B). Glucuronide conjugates of (-)-borneol and TMA were undetectable during the present HPLC analyses.

To minimize the potential degradation of 2-PPA-CoA and 2-PPA-1-O-G during processing of liver tissue during homogenization, we carried out homogenization experiments under acidic conditions (pH 5), on ice (−4°C), and over a short time period (less than 40 s). The resultant liver homogenates were immediately denatured to prevent any potential enzymatic degradation of 2-PPA-CoA and 2-PPA-1-O-G derivatives. HPLC analysis was performed immediately after sample preparation. Preliminary studies with synthetic 2-PPA-CoA and biosynthetic 2-PPA-1-O-G showed that 2-PPA-CoA thioester and 2-PPA-1-O-G were stable under these extraction conditions.

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**Fig. 2.** Representative reverse-phase HPLC analysis of 2-PPA-CoA from rat liver extracts dosed with (R,S)-2-PPA. Rats were pretreated i.p. with corn oil (control, 1 ml/rat i.p.) (A), (-)-borneol (320 mg/kg in corn oil, 1 ml/rat) (B), or TMA (500 mg/kg in corn oil, 1 ml/rat) (C) 30 min before receiving (R,S)-2-PPA (130 mg/kg in 0.9% saline i.p., 0.5 ml/rat). Rats were then sacrificed 2 h after 2-PPA administration. Their livers were collected, processed, and analyzed by HPLC.
Borneol inhibition studies were carried out with a (R,S)-2-PPA metabolism. Borneol pretreatment regimen with minor modifications of previously described procedures (Watkins and Klaassen, 1982). Hundred and ninety-nine (199). Preliminary studies with (R,S)-2-PPA showed that a high dose of (R)-borneol (750 mg/kg in corn oil) (Watkins and Klaassen, 1982, 1983) caused acute CNS effects in rats. Ten minutes postadministration of (R)-borneol, rats seemed uncoordinated and motor activity was markedly reduced. The toxic symptoms were transient and the (R)-borneol-treated rats seemed to completely recover 1 h after dosing. To avoid high-dose CNS effects of (R)-borneol, we conducted dose-dependent inhibition studies (0, 160, 320, and 480 mg/kg) to determine the lowest effective inhibitory dose. The inhibition of 2-PPA acyl glucuronidation by (R)-borneol was dose-dependent. 2-PPA acyl glucuronidation was completely inhibited by the 320- and 480-mg/kg doses of (R)-borneol without demonstrating the acute CNS effects noted above. Therefore, a 320-mg/kg dose of (R)-borneol was chosen for the further characterization of its inhibitory effects on 2-PPA acyl glucuronidation, acyl-CoA formation, and covalent binding to protein.

To determine the optimal dose of TMA that can effectively inhibit 2-PPA-CoA formation, rats were pretreated with various doses of TMA in corn oil 10 min before 2-PPA administration. Compared with 0.9% saline pretreatment, 2-PPA-CoA formation was inhibited substantially (41%) by corn oil alone (Fig. 4), which had no effect on 2-PPA acyl glucuronidation (data not shown). The combination of corn oil with TMA resulted in further decreases of 2-PPA-CoA formation in rat livers. A 500 mg/kg TMA dose in corn oil (1 ml) was found to be the optimal, inhibiting 2-PPA-CoA formation by 86% (Fig. 4), whereas further increases of the TMA dose did not result in a greater decrease of 2-PPA-CoA formation. Therefore, 500 mg/kg TMA in corn oil was chosen for the covalent binding studies.

**Inhibitory Studies with (R,S)-2-PPA.** In control rats (corn oil-pretreated), the formation of 2-PPA-CoA was very rapid and achieved an apparent plateau (49 nmol/g liver) 0.25 h postadministration of (R,S)-2-PPA (Fig. 5A). 2-PPA acyl glucuronidation [sum of (R)- and (S)-2-PPA-1-O-G isomers] in control rats was evident after 0.25 h and achieved a maximum of 632 nmol/g liver 0.5 h postadministration of (R,S)-2-PPA (Fig. 6A). The maximum level of 2-PPA acyl glucuronide in livers from (R,S)-2-PPA-pretreated rats was nearly 12-fold greater than maximum level of 2-PPA-CoA formation (Figs. 5A and 6A), which is consistent with the higher capacity of acyl glucuronidation enzymes.

The formation of 2-PPA-CoA thioester in (S)-borneol-pretreated rats showed a similar concentration-time dependent profile to that of control rats (corn oil-treated), with 44 nmol/g liver concentration measured at 0.25 h after 2-PPA administration (Fig. 5B). Conversely, 2-PPA acyl glucuronidation was markedly inhibited by (S)-borneol-pretreatment (Fig. 6B). 2-PPA acyl glucuronides were undetectable by HPLC in (S)-borneol-pretreated rats during the first 1 h after 2-PPA administration. The concentration of 2-PPA acyl glucuronides detected in (S)-borneol-pretreated rats was 110 ± 109 nmol/g liver at the 2-h time point, which was significantly lower than that determined in control rat livers (543 ± 48 nmol/g liver) (Fig. 6A). In (S)-borneol-treated rats 24 h after 2-PPA administration, the exposure [AUC(0-24 h)] of 2-PPA acyl glucuronides to liver proteins over a 2-h period was markedly decreased by (S)-borneol (Table 1, 95%). Note that contrary to general belief, acyl glucuronide concentrations can approximate concentrations of the parent aglycone when glucuronidation is not inhibited (Fig. 5A, B, and C).

2-PPA-CoA formation in TMA-treated rats was detected over the 2-h period, but with much lower concentrations measured compared with that determined in control rat liv-

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**Fig. 3.** Representative reverse-phase HPLC analysis of 2-PPA acyl glucuronides from rat liver extracts dosed with (R,S)-2-PPA. Rats were pretreated i.p. with corn oil (control, 1 ml/rat) (A), (−)-borneol (320 mg/kg in corn oil, 1 ml/rat) (B), or TMA (500 mg/kg in corn oil, 1 ml/rat) (C) 30 min before receiving (R,S)-2-PPA (130 mg/kg in 0.9% saline, i.p., 0.5 ml/rat). Rats were then sacrificed 2 h after 2-PPA administration. Their livers were collected, processed, and analyzed by HPLC.

**Fig. 4.** Dose-dependent inhibition of 2-PPA-CoA formation by TMA. Rats were pretreated with 0.9% saline (1 ml/rat i.p.), corn oil (1 ml/rat i.p.), and increasing concentrations of TMA in corn oil (1 ml/rat i.p.) 10 min before 2-PPA administration (130 mg/kg in 0.9% saline, 0.5 ml/rat i.p.). One rat was used for each dosage treatment.
ers (Fig. 5C). TMA-pretreatment markedly decreased 2-h AUC values for 2-PPA-CoA (64%) from control (Table 1). In contrast, the formation of 2-PPA acyl glucuronides in TMA-treated rats showed a similar concentration-time dependent profile to control rats (Fig. 6; Table 1).

The effects of (-)-borneol and TMA on the metabolic activation of 2-PPA and the extent of covalent binding of 2-PPA to liver proteins was further examined 2 h after (R,S)-[1-14C]2-PPA administration. As shown in Table 1, the levels of 2-PPA-CoA and acyl glucuronides in livers from rats 2 h postadministration of (R,S)-[1-14C]2-PPA were consistent with those levels 2 h after nonradiolabeled (R,S)-2-PPA treatment (Figs. 5 and 6). Due to the limited availability of (R,S)-[1-14C]2-PPA in our laboratory, time-dependent studies with (R,S)-[1-14C]2-PPA were not performed. However, the metabolite concentration-time profiles (AUC) obtained from nonra-

Table 1
Effects of (-)-borneol and TMA on 2-PPA-CoA formation, acyl glucuronidation, and covalent binding to protein in vivo in rat liver.

<table>
<thead>
<tr>
<th></th>
<th>Covalent Binding</th>
<th>AUC 2 h Post-administration (Treated/Control)</th>
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<tbody>
<tr>
<td></td>
<td>pmol/mg protein</td>
<td>2-PPA-CoA</td>
</tr>
<tr>
<td>Control</td>
<td>150 ± 5</td>
<td>50.0 ± 8.5</td>
</tr>
<tr>
<td>(-)-Borneol-treated</td>
<td>115 ± 10b</td>
<td>46.8 ± 4.3</td>
</tr>
<tr>
<td>TMA-treated</td>
<td>75.8 ± 5.3c</td>
<td>12.4 ± 1.9c</td>
</tr>
</tbody>
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- Rats were pretreated i.p. with 1) corn oil (1 ml/rat); 2) (-)-borneol (320 mg/kg in corn oil, 1 ml/rat); 3) trimethylacetic acid (TMA, 500 mg/kg in corn oil, 1 ml/rat), 30 min before receiving an i.p. injection of (R,S)-[1-14C]2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Livers were collected 2 h after (R,S)-[1-14C]2-PPA administration. Values are expressed as the mean ± S.D. (n = 4).
- Significantly different than control using Student’s t/Newman-Keuls method.
- Significantly different than (-)-borneol-treated using Student’s t/Newman-Keuls method.
As indicated in Table 1, pretreatment with (−)-borneol, which markedly inhibited the exposure of 2-PPA acyl glucuronide by 95%, only decreased covalent binding by 23% (p < 0.05) 2 h postadministration of (R,S)-[1-14C]2-PPA. Conversely, covalent binding of 2-PPA to liver proteins decreased by 49% (p < 0.05) in rats pretreated with TMA. Even though cost constraints and the time-consuming aspects of covalent binding studies only allowed us to examine the dependence of 2-PPA-CoA and 2-PPA acyl glucuronide conjugate formation in two rats per time point per condition, it seems obvious that the 49% decrease in covalent binding in TMA-pretreated rats better approximates the change in 2-PPA-CoA hepatic exposure, which decreased 64%, than the hepatic exposure to 2-PPA acyl glucuronide, which decreased 1% (Table 1).

Discussion

Chemically reactive intermediates formed during the metabolism of profen drugs are believed to mediate their toxic side effects by binding covalently to proteins (Boelsterli et al., 1995; Pumford and Halmes, 1997). Two alternative metabolic activation pathways involved in the generation of reactive acylating metabolites of profen drugs are acyl glucuronidation and acyl-CoA formation (Fig. 1). Acyl glucuronides have been well documented over the past two decades to be intrinsically reactive electrophiles that can bind covalently to serum albumin in vitro and to plasma and tissue protein in vivo (Spahn-Langguth and Benet, 1992; Benet et al., 1993; Hayball, 1995; Li and Benet, 2002). Acyl-CoA thioester derivatives, on the other hand, are only recently being increasingly recognized as reactive metabolites of acidic drugs. S-Acyl-CoA thioesters of xenobiotic carboxylic acids, e.g., clofibric acid (Grillo and Benet, 2002), 2-PPA (Li et al., 2002b), naproxen (Olsen et al., 2002), and 2,4-dichlo-rophenoxycetic acid (Li et al., 2003), are chemically reactive species that can readily transacylate the GSH sulphydryl group and protein nucleophiles. Because both metabolic activation pathways may mediate the covalent binding of profen drugs to proteins, caution should be taken in concluding the contribution of a single metabolic activation pathway to covalent adduct formation. The present studies were designed to determine the relative contribution of 2-PPA acyl glucuronidation and 2-PPA-CoA formation on the 2-PPA-protein covalent adduct formation in vivo in 2-PPA-dosed rats. 2-PPA was chosen because it is the simplest congener of the profen class of nonsteroidal anti-inflammatory drugs and also because it is known to be metabolized in rats primarily by acyl glucuronidation and acyl-CoA formation.

Inhibition studies with 2-PPA in vivo in rats showed that selective inhibition of each metabolic pathway led to a decrease in covalent binding of 2-PPA to liver proteins (Table 1), indicating that both metabolic pathways, acyl glucuronidation and acyl-CoA formation, are involved in covalent binding. The extent of covalent binding of 2-PPA to liver proteins in vivo was markedly decreased (49%, p < 0.05) when the hepatic exposure of acyl-CoA thioester was inhibited 64% by TMA pretreatment (Table 1; Fig. 5). In contrast, inhibition of acyl glucuronidation by 95% by (−)-borneol only decreased covalent adduct formation by 23% (Table 1; Fig. 6). These results strongly suggest that metabolic activation by 2-PPA-CoA formation contributes to covalent adduct formation to protein in vivo to a greater extent than metabolic activation by acyl glucuronidation, even though the level of 2-PPA-acyl glucuronides was much greater than that of 2-PPA-CoA thioester in liver extracts from 2-PPA-dosed rats (Figs. 5 and 6; Table 1). The relative contribution of these two metabolic pathways to covalent binding depends not only on the exposure of reactive metabolite to liver proteins but also on the relative reactivity of the metabolites toward proteins. A higher contribution of the acyl-CoA pathway to covalent adduct formation therefore presumably results from the higher chemical reactivity of acyl-CoA thioester derivatives with protein nucleophiles, compared with that of acyl glucuronides. In fact, we have shown that 2-PPA-CoA thioester was approximately 70-fold more reactive with the cysteiny1 sulphydryl of GSH (a model nucleophile) forming 2-PPA-S-acyl glutathione in vitro in buffer compared with reactions with 2-PPA-1-O-G (Li et al., 2002b).

(−)-Borneol, a monoterpenoid alcohol, has been widely used as an inhibitor for acyl glucuronidation both in vitro in hepatocytes (Porubek et al., 1989; Kretz-Rommel and Boelsterli, 1993) and in vivo in rats (Watkins and Klaassen, 1982; Hong et al., 1999). A dose range of 750 to 900 mg/kg (−)-borneol was used in previous in vivo inhibition studies (Watkins and Klaassen, 1982; Hong et al., 1999). Our preliminary studies showed that at a dose of 750 mg/kg (−)-borneol caused acute CNS effects in rats. To avoid the CNS effects associated with exposure to (−)-borneol, we conducted dose-dependent inhibition studies to determine the lowest effective inhibitory dose of (−)-borneol on 2-PPA glucuronidation not causing untoward CNS effects. A significantly lower dose of (−)-borneol, 320 mg/kg, was chosen in our in vivo inhibition studies because (−)-borneol effectively inhibited 2-PPA acyl glucuronidation (Fig. 3) without demonstrating CNS effects in rats. Acyl glucuronidation of 2-PPA in rat livers was decreased by 95% at a 320-mg/kg dose of (−)-borneol, whereas the extent of covalent binding of 2-PPA to liver proteins was decreased by only 23%, suggesting that 2-PPA acyl glucuronidation is a contributor, but not the major factor, in the covalent binding of 2-PPA to protein in vivo.

Dose-dependent inhibition studies showed that corn oil itself inhibited 2-PPA-CoA formation by 41% (Fig. 4), but had little effect on 2-PPA acyl glucuronidation (data not shown). The main ingredients of corn oil are glycerides of long-chain fatty acids, which are quickly hydrolyzed to glycol and long-chain fatty acids in the liver. Because long-chain fatty acids are substrates for acyl-CoA formation, competitive inhibition of acyl-CoA synthetases, and therefore 2-PPA-CoA formation, might occur. Therefore, it is important to consider the vehicle effect on drug metabolism when performing in vivo studies. When rats were dosed with corn oil and TMA together, a further decrease of 2-PPA-CoA formation was observed.

TMA, also known as pivalic acid, is a small branched chain carboxylic acid, widely used for prodrug production to improve oral bioavailability, e.g., pivampicillin (Binderup et al., 1971) and pivaloxyloxymethyl dopa ester (Vickers et al., 1984). Studies on the biological fate of TMA revealed that TMA acyl glucuronide, TMA-glycine, and TMA-carnitine conjugates were the major urinary metabolites in vivo in rat urine (Mizojiri et al., 1995). TMA-CoA is believed to be formed in vivo in rats because it is the obligatory intermediate for glycine and carnitine conjugates of TMA. Studies with TMA in hepatocytes provided direct evidence of the capability of diolabeled (R,S)-2-PPA studies seem to be a good estimate of the exposure of reactive metabolites of [1-14C]2-PPA to liver proteins in (R,S)-[1-14C]2-PPA-treated rats.
rat liver cells to catalyze the formation of TMA-CoA thioester (Ruff and Brass, 1991). Consistent with the above-mentioned literature, TMA-CoA thioester was detected by HPLC analysis of liver extracts from TMA-pretreated rats (Fig. 2C). Because both acyl glucuronide and acyl-CoA thioester derivatives of TMA are formed in vivo, it is interesting, and advantageous that TMA selectively inhibited 2-PHA-CoA formation by 64%, but not 2-PHA acyl glucuronidation (Table 1). As a result of these changes in metabolic activation of 2-PHA, pretreatment with TMA led to a 49% decrease in covalent binding of 2-PHA to liver proteins (Fig. 5; Table 1), suggesting that 2-PHA-CoA formation is the major pathway for covalent binding of 2-PHA to hepatic protein.

The results from these in vivo inhibition studies are consistent with in vitro covalent binding studies performed in rat hepatocytes, where it was shown that the covalent binding of 2-PHA to hepatic protein exhibited a 53% decrease in cells treated with trimethylacetic acid, where a 66% decrease in 2-PHA-CoA formation occurred, but that treatment with (−)-borneol, which completely inhibited 2-PHA acyl glucuronidation, only decreased covalent binding by 18.7% (Li et al., 2002a). Further evidence indicating the importance of covalent binding of acyl-CoA formation on irreversible 2-PPA-protein binding came from studies showing that the enantioselectivity of covalent binding correlated with the enantioselectivity of acyl-CoA formation (R/S = 7.0), but not with acyl glucuronidation (R/S = 0.67) of R- and S-2-PHA isomers in incubations with rat hepatocytes (Li et al., 2002a).

In conclusion, this is the first in vivo study performed to compare the contribution of acyl glucuronidation and acyl-CoA formation to acidic drug-protein covalent adduct formation. By directly monitoring reactive metabolite levels and covalent binding to proteins in rat livers, we have demonstrated that metabolic activation of 2-PHA by 2-PHA-CoA formation contributes to 2-PHA covalent adduct formation to protein in vivo to a greater extent than metabolic activation by 2-PHA acyl glucuronidation. Ongoing studies in our laboratory are designed to compare the relative importance of these two pathways for other carboxylic acid-containing drugs and xenobiotics.

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References

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