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Enantioselective Covalent Binding of 2-Phenylpropionic Acid to Protein in Vitro in Rat Hepatocytes†

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A series of studies was conducted to investigate the potential of (*R*)- and (*S*)-2-phenylpropionic acid (2-PPA) to undergo enantioselective covalent binding to protein in freshly isolated rat hepatocytes and to determine whether such covalent binding is dependent on acyl glucuronidation or acyl-CoA formation of 2-PPA. Hepatocytes were incubated with (*R*,*S*)-, (*R*)-, or (*S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM), and aliquots of the incubation mixture analyzed for covalent binding, acyl glucuronidation, and acyl-CoA formation over a 3 h period. Covalent binding of 2-PPA to hepatocyte protein was shown to be time-dependent and to be 4.5-fold greater for the (*R*)-isomer than the (*S*)-isomer after 3 h of incubation. 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-PPA isomers during the 3 h incubation. Inhibition experiments were performed with (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) incubated with hepatocytes in the presence or absence of trimethylacetic acid (2 mM) or (−)-borneol (1 mM) for the inhibition of 2-PPA-CoA formation and 2-PPA acyl glucuronidation, respectively. Covalent binding of 2-PPA to hepatocyte protein exhibited a 53% decrease in cells treated with trimethylacetic acid, where a 66% decrease in 2-PPA-CoA formation occurred. Conversely, treatment with (−)-borneol, which completely inhibited 2-PPA acyl glucuronidation, only decreased covalent binding by 18.7%. These results indicate that metabolism of 2-PPA by acyl-CoA formation leads to the generation of reactive acylating CoA-thioester species that can contribute to protein covalent binding in a manner that is more extensive than the respective acyl glucuronides.

Introduction

2-Arylpropionic acids are a widely used class of nonsteroidal antiinflammatory drugs (NSAIDs).[1] These compounds have a chiral center at the carbon alpha to the carboxylic acid, and only the (*S*)-enantiomers exhibit significant antiinflammatory activity (*1*). Nevertheless, the clinically used 2-arylpropionic acids are marketed as racemates with the notable exception of (*S*)-naproxen. Conjugation with glucuronic acid is a major route for the biotransformation and elimination of these drugs, such as ibuprofen, carprofen, ketoprofen, and fenoprofen (*2*). Acyl-linked glucuronides have been shown to be reactive electrophilic metabolites, capable of undergoing hydrolysis to regenerate the pharmacological active parent drug and intramolecular rearrangement to yield β-glucuronidase resistant isomers (*3*). More importantly, these electrophilic metabolites can bind covalently to proteins both in vitro and in vivo. The in vivo formation of covalently bound plasma protein adducts by acyl glucuronides has been demonstrated in humans for a large number of compounds, including tolmetin (*4*, *5*), zomepirac (*6*), carprofen (*7*), diflunisal (*8*), fenoprofen (*9*), and ketoprofen (*10*). In vitro studies also demonstrated that tolmetin acyl glucuronide (*11*) and diclofenac acyl glucuronide (*12*) directly contributed to the formation of tissue protein adducts. These in vitro studies indicate the potential involvement of acyl glucuronides in the covalent binding of acidic drugs to tissue proteins in vivo, which may help explain some of drug-induced organ toxicity associated with carboxylic-acid-containing drugs (*3*).

Another remarkable feature of the metabolism of Profen drugs is the unidirectional chiral inversion from the pharmacologically inactive (*R*)- to the active (*S*)-enantiomer. Such inversion has been documented by a large number of in vivo studies using a variety of species and 2-arylpropionic acid drugs (*13*). The mechanism of this inversion reaction is believed to involve the initial enantioselective formation of acyl-coenzyme A (acyl-CoA) thioester followed by epimerization and finally hydrolysis to regenerate free acids (Figure 1). For each 2-arylpropionic acid drug studied, almost no acyl-CoA formation was observed for the (*S*)-enantiomers, while the respective acyl-CoA thioester derivatives were readily detected for most (*R*)-enantiomers. Such enantioselective formation of acyl-CoA thioesters is consistent with the known enantioselectivity, substrate and species selectivity of chiral inversion in vivo (*14*).

In addition to mediating the chiral inversion of 2-arylpropionic acid drugs, activated acyl-CoA thioesters also serve as obligatory intermediates for the formation of

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[1] Abbreviations: NSAIDs, nonsteroidal antiinflammatory drugs; 2-PPA, 2-phenylpropionic acid; 2-PPA-CoA, 2-phenylpropionyl-*S*-acyl-CoA; 2-PPA-1-*O*-G, 2-phenylpropionyl-1-*O*-acyl glucuronide; CoA, coenzyme A; TMA, trimethylacetic acid; ESI, electrospray ionization; CID, collision-induced dissociation.

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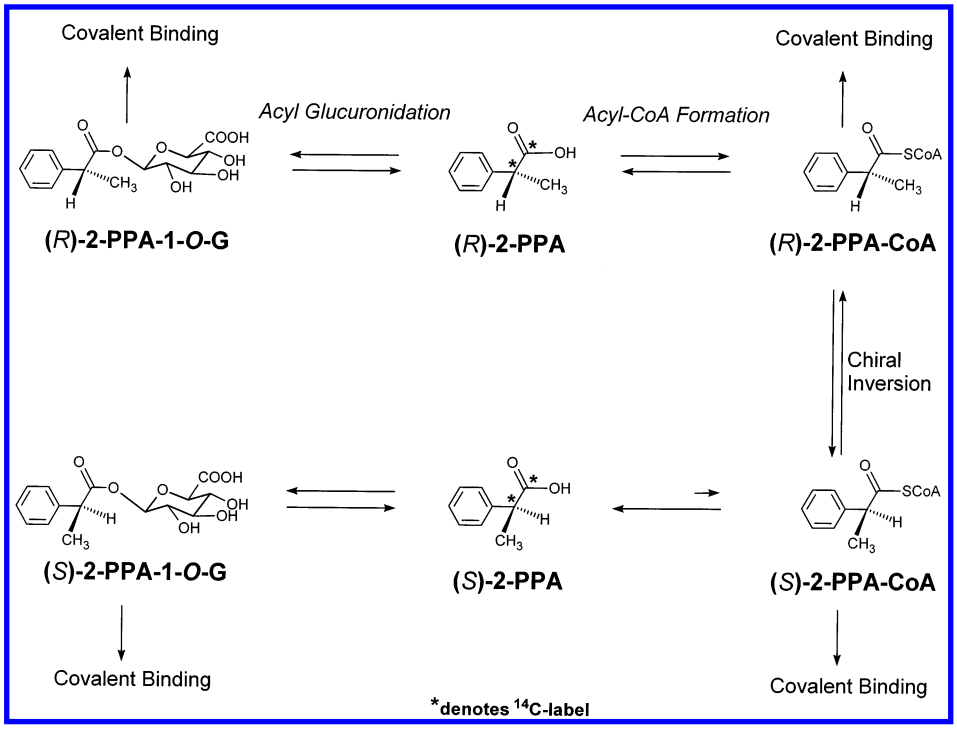

Figure 1. Scheme for the metabolic activation of (*R*)- and (*S*)-2-phenylpropionic acid by acyl-CoA formation and acyl glucuronidation.

amino acid conjugates, carnitine esters, and hybrid triglycerides. Formation of thioester-linked acyl-CoA thioesters increases the chemical electrophilicity of carboxylic acids. It has been shown that long-chain fatty acyl-CoAs, such as palmitoyl-CoA and myristoyl-CoA, spontaneously acylate sulfhydryl groups on proteins or peptides in vitro in a time- and concentration-dependent fashion (*15*, *16*). In addition, it was demonstrated that covalent binding of nafenopin to human liver homogenate proteins required prior formation of a nafenopin acyl-CoA thioester intermediate (*17*). Our recent studies (*18*) have shown that acyl-CoA of 2-phenylpropionic acid (2-PPA) was a better transacylation derivative than the 1-*O*-acyl glucuronide of 2-PPA in reactions with glutathione (GSH). It was shown that 2-PPA-CoA readily underwent a rapid transacylation-type reaction with GSH in buffer (pH 7.4 and 37 °C), while 2-PPA 1-*O*-acyl glucuronide (2-PPA-1-*O*-G) mainly underwent intramolecular acyl migration, rather than reacting with GSH. These findings suggest that xenobiotic acyl-CoA thioesters are reactive electrophiles that may contribute to covalent binding of acidic drugs to protein.

Acyl glucuronidation and acyl-CoA formation are two common metabolic pathways for the 2-arylpropionic acid drugs. Both pathways allow for the formation of reactive metabolites, which may lead to covalent binding of acidic drugs to proteins (Figure 1). The chemical reactivity of acyl glucuronides of acidic NSAIDs toward proteins has been extensively studied (*2*). In contrast, limited data has been published to illustrate the potential involvement of xenobiotic acyl-CoA thioesters in covalent binding. The relative importance of these two metabolic pathways for covalent binding remains unresolved. The present studies were designed to examine the relative contribution of these two metabolic activation pathways to the covalent binding of 2-PPA (a model compound of 2-arylpropionic acid drugs) to protein in hepatocytes. We propose that if 2-PPA-CoA is more important than 2-PPA-1-*O*-G in the covalent binding to protein in vitro, then incubations with the (*R*)-isomer of 2-PPA should lead to greater covalent binding to protein compared to incubations with the (*S*)-isomer. By using two independent approaches, i.e., (1) inhibition of 2-PPA acyl-CoA formation and acyl glucuronidation and subsequent effects on covalent binding of ^{14}C-labeled 2-PPA to rat hepatocyte protein in vitro, and (2) enantioselective studies with ^{14}C-labeled (*R*)- and (*S*)-2-PPA, we demonstrate that the acyl-CoA thioester formation of 2-PPA plays a more important role for covalent binding to protein than its acyl glucuronide in vitro in rat hepatocytes, at least for this compound.

Experimental Section

Chemicals. (*R*,*S*)-2-PPA, (*R*)-2-PPA, and (*S*)-2-PPA were purchased from Aldrich Chemical Co. (Milwaukee, WI). Trimethylacetic acid (TMA) and (−)-borneol were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA, 2-PPA-CoA, and biosynthetic 2-PPA-1-*O*-G were obtained from previous studies in this laboratory (*18*). Purified (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were obtained as described below. All solvents used for HPLC were of chromatographic grade.

Purification of (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA. (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were purified from 50 mg of synthetic (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA (0.1 mCi/mmol, dissolved in 1 mL of mobile phase) by reversed-phase isocratic HPLC of 12.5 μL aliquots using 0.1% trifluoroacetic acid in 30% acetonitrile on a chiral column (Chiralcel OJ-R, 4.6 × 150 mm, Chiral Technologies Inc., Exton, PA) at a flow rate of 0.8 mL/min and with UV detection at 226 nm. Fractions isolated from HPLC purification and that contained (*R*)- or (*S*)-[1,2-$^{14}C_2$]-2-PPA were combined and the acetonitrile removed by evaporation under reduced pressure. The remaining aqueous phase (∼50 mL) was acidified (pH 3.0) and extracted with ethyl ether (3 × 50 mL) until no significant radioactivity was detected in the aqueous phase. The combined ethyl ether extracts were evaporated under reduced pressure and at room temperature to yield a clear, colorless oil (∼80% yield of each resolved enantiomer). The identities of the resolved (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were confirmed by coelution on HPLC (as above) with commercially available (*R*)- and (*S*)-2-PPA standards (data not shown) and by ^{1}H NMR analysis (which showed identical spectra for each enantiomer). ^{1}H NMR

(deuteriochloroform): δ 7.27−7.38 (m, 5H, phenyl ring), 3.74−3.80 (q, 1H, -CH-), 1.54−1.56 (d, 3H, $-CH_3$). The enantiomeric purities of (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were estimated, by HPLC, to be 86% enantiomeric excess of (*R*)-2-PPA and 96% enantiomeric excess of (*S*)-2-PPA, respectively. Both (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were determined to be greater than 99% radiochemically pure as assessed by scintillation counting of HPLC fractions (0.1 mL) that were collected every 30 s by chromatography as described above.

In Vitro Studies with Rat Hepatocytes. Freshly isolated rat hepatocytes were prepared from a single rat (300 g, male Sprague−Dawley) according to published procedures (*19*) and greater than 85% viability was achieved, as assessed by trypan blue exclusion. Incubations of hepatocytes (4 million cells/mL) with (*R*,*S*)-, (*R*)-, or (*S*)-[1,2-$^{14}C_2$]-2-PPA (0.1 mCi/mmol, 1.0 mM) were performed in Krebs-Henseleit buffer (pH 7.4) in 50 mL round-bottom flasks with continuous rotation and gassed with 95% O_2/5% CO_2 at 37 °C. Aliquots of the incubation were taken at 2, 5, 10, 15, 20, 30, 60, 120, and 180 min and analyzed for covalent binding to protein, acyl glucuronidation, and acyl-CoA formation. For inhibition experiments, inhibitors of metabolism, namely TMA (inhibitor of acyl-CoA formation, final concentration 2 mM) or (−)-borneol (inhibitor of glucuronidation, final concentration 1 mM), were added to hepatocyte incubations and a further 5 min period was allowed before the addition of (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA (final concentration 1 mM). The effects of these inhibitors on covalent binding, acyl-CoA formation, and acyl glucuronidation of 2-PPA were assessed at the 3 h time point after the addition of (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA to the incubation mixture. Stock solutions of TMA, (*R*,*S*)-, (*R*)-, and (*S*)-[1,2-$^{14}C_2$]-2-PPA were prepared as 20 mM solutions in water (pH 7.0). A stock solution of (−)-borneol was prepared as a 100 mM solution dissolved in absolute ethanol. For enantioselective studies, freshly isolated rat hepatocytes were incubated with (*R*)- or (*S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) and aliquots of the incubation mixture analyzed for covalent binding to protein, acyl glucuronidation, and acyl-CoA formation in a time-dependent fashion over a 3 h period (as above).

After the addition of 2-PPA free acid to hepatocyte incubations, aliquots (3 mL) of the incubation mixture were taken at indicated times and quenched with perchloric acid (7%, 1.5 mL) for determination of acyl-CoA and covalent binding to protein. After centrifugation (2500 rpm, 10 min), the supernatants were neutralized with 1 N NaOH and analyzed by reversed-phase HPLC. Covalent binding of 2-PPA to protein was measured by scintillation counting of exhaustively washed hepatocyte protein precipitates. Briefly, protein precipitates were washed five times with 7% $HClO_4$/phosphate buffer, pH 7.4 (1:2, v/v), eight times with ethanol/diethyl ether (3:1, v/v), followed by eight times with MeOH/H_2O (4:1, v/v). The remaining protein pellets were dissolved by heating in 1 N NaOH (0.65 mL) at 80 °C for 1 h. Six hundred microliters of the hydrolyzed protein solution was added to 600 μL of H_2SO_4 (3 N), followed by the addition of 10 mL of scintillation fluid (Hionic-Fluro, Packard BioScience Company, Meriden, CT), vortex mixed, and analyzed by scintillation counting. Protein concentrations were determined in a 5 μL aliquot of the hydrolyzed protein pellet solution using BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as the standard, following the manufacturer's instructions. Covalent binding is expressed as picomoles of 2-PPA bound per milligram of protein.

For the analysis of 2-PPA acyl glucuronidation in hepatocytes, 0.5 mL aliquots from the hepatocyte incubations were taken at indicated time points and quenched with acetonitrile (0.5 mL) and HCl (1 N, 12.5 μL). After centrifugation (14 000 rpm, 10 min), supernatants were analyzed by reversed-phase HPLC to quantify 2-PPA acyl glucuronidation. Quantitative measurements of 2-PPA-CoA and 2-PPA-acyl glucuronide formation were made using a standard curve generated from absolute peak areas.

To determine if cell viability and functional enzymes were necessary for covalent binding, incubations were performed, as above, with (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA and with hepatocytes that were heat-denatured by immersing incubation flasks containing cells into heated water (90 °C) for 3 min, followed by a 5 min cooling period, prior to incubations.

HPLC Analysis. Briefly, incubations were analyzed for 2-PPA-acyl-CoA and 2-PPA-1-*O*-G as follows. Analysis of 2-PPA-CoA was performed on a C8 Zorbax column (150 × 4.6 mm, 5 μm, MAC-MOD Analytical, Chadds Ford, PA) at a flow rate of 1.0 mL/min. The isocratic mobile phase contained 17.5% acetonitrile in 0.19 M ammonium acetate buffer (pH 7.0) and analysis was performed with UV detection at 262 nm. Analysis for 2-PPA-acyl glucuronide was performed by isocratic elution 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. The mobile phase contained 0.1% TFA in 15% acetonitrile and UV detection was at 226 nm.

Mass Spectrometry. Mass spectrometric analysis was performed on (*R*)- and (*S*)-2-PPA-CoA obtained by HPLC purification of extracts from respective incubations of (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA treated hepatocytes. Analysis was performed on synthetic standard and HPLC purified, and dried, extracts using a Hewlett-Packard HP 1100 LC/MSD benchtop electrospray mass spectrometer (Palo Alto, CA) in positive ion mode and at a fragmentor voltage of 130 by direct infusion of the sample (dissolved in 10 mM ammonium acetate, pH 5.0/acetonitrile, 1/1) into the ion source (500 μL/min). A Finnigan-MAT TSQ 7000 (San Jose, CA) was used for the mass spectrometric analysis of HPLC purified 2-PPA acyl glucuronide with analysis in the negative ion mode and by CID of the deprotonated molecular ion $[M - H]^-$ at *m*/*z* 325. Analysis of standard (*R*,*S*)-2-PPA-1-*O*-G provided a mass spectrum by this method having the following product ions: *m*/*z* 193 ([glucuronic acid]$^-$, 56%), *m*/*z* 175 ($[C_6H_7O_6]^-$, 25%), *m*/*z* 149 ($[C_9H_9O_2]^-$, 37%), *m*/*z* 113, 100%. Samples were analyzed by direct infusion into the ion source using the mobile phase described above for the analysis of 2-PPA-CoA.

Results

HPLC Analysis of 2-PPA-CoA and 2-PPA Acyl Glucuronide from Extracts of Rat Hepatocytes. The analysis of 2-PPA-CoA formed during incubations of (*R*)- or (*S*)-2-PPA with freshly isolated rat hepatocytes was performed by reversed-phase HPLC of perchloric acid extracts with isocratic elution and UV-detection at 262 nM (the absorbance maximum for CoA). Results showed that both (*R*)- and (*S*)-2-PPA form 2-PPA-CoA having an approximate retention time of 5.5 min and that they coeluted with synthetic (*R*,*S*)-2-PPA-CoA standard (data not shown). Incubations performed in the absence of 2-PPA yielded extracts that upon HPLC analysis showed no peak eluting at the retention time of 2-PPA-CoA (data not shown). The identity of 2-PPA-CoA formed in the (*R*)- and (*S*)-2-PPA-treated hepatocyte incubations was confirmed by positive ion ESI/MS of HPLC-purified biological extracts and provided a mass spectrum characteristic of 2-PPA-CoA synthetic standard, which included ions at *m*/*z* 291, 330, 393, 410, 428, 491, and 609 originating from the proposed cleavages shown in Figure 2.

The analysis of 2-PPA acyl glucuronides formed during hepatocyte incubations in the presence and absence of 2-PPA (1 mM) was also performed by reversed-phase isocratic HPLC (Figure 3), which allowed for the separation of (*R*)- and (*S*)-2-PPA-1-*O*-acyl glucuronides, eluting at 10.9 and 11.6 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 of (*R*)- or (*S*)-2-PPA were observed during the HPLC analysis of control hepatocyte incuba-

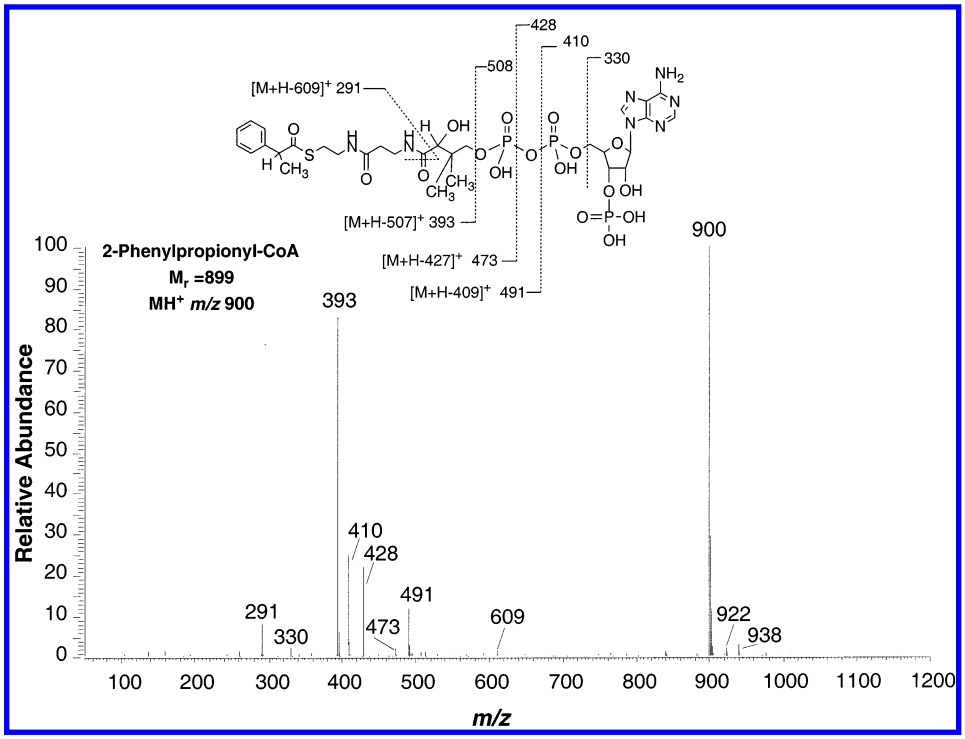

Figure 2. ESI/MS positive ion mass spectrum of in vitro formed 2-PPA-CoA obtained from extracts of the incubation of (*R*)-2-PPA (1 mM) with freshly isolated rat hepatocytes (4 million cells/mL) after 0.5 h of incubation. The origins of the characteristic fragments are as shown.

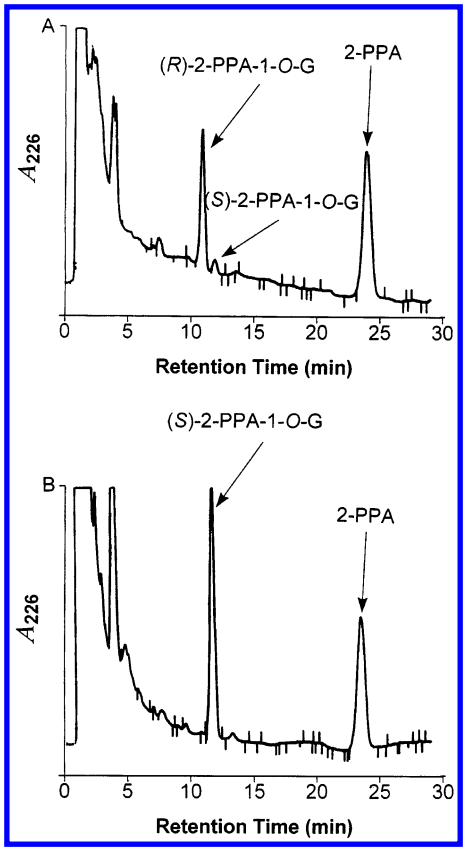

Figure 3. Representative reversed-phase HPLC analysis of 2-PPA-1-*O*-G from extracts of rat hepatocytes incubated with (A) (*R*)-2-PPA or (B) (*S*)-2-PPA.

tion extracts (data not shown). The identities of the resolved and purified (*R*)- and (*S*)-2-PPA-1-*O*-G were confirmed by HPLC analysis of their alkaline hydrolysis and β-glucuronidase hydrolysis products compared to purchased commercial (*R*)-2-PPA and (*S*)-2-PPA. The identity of (*R*)- and (*S*)-2-PPA-1-*O*-G formed in the (*R*)- and (*S*)-2-PPA-treated hepatocyte incubations was confirmed by negative ion tandem mass spectrometry. CID of the parent $[M - H]^-$ ion (*m*/*z* 325) of HPLC-purified biological extract provided a product ion mass spectrum characteristic of 2-PPA-1-*O*-G synthetic standard, which included ions at *m*/*z* 45, 59, 105, 113, 149, 175, and 193 originating from the proposed cleavages shown in Figure 4.

Time Course of 2-PPA Acyl-CoA Formation, Acyl Glucuronidation, and Covalent Binding in Isolated Rat Hepatocytes. When freshly isolated rat hepatocytes were incubated with (*R*,*S*)-$^{14}C_2$-2-PPA (1 mM), covalent binding to protein was shown to be time-dependent and reached a maximum plateau of nearly 230 pmol of 2-PPA bound/mg of protein after 1 h of incubation (Figure 5A). The formation of 2-PPA-CoA was very rapid and reached a maximum concentration of ~0.93 nmol/million cells at the 15 min time point (Figure 5B), while 2-PPA acyl glucuronidation occurred in a time-dependent and nearly linear fashion over 3 h of incubation, reaching ~40 nmol/million cells at the 3 h time point (Figure 5C). No 2-PPA-acyl glucuronide could be detected by HPLC with UV-detection before the 15 min time point. No covalent adduct formation, 2-PPA-CoA formation nor 2-PPA acyl glucuronidation was observed in incubations of heat-denatured hepatocytes treated with (*R*,*S*)-$^{14}C_2$-2-PPA (1 mM) (data not shown). The maximum level of 2-PPA acyl glucuronide formed (3 h time point) was nearly 40-fold higher than that of 2-PPA-CoA formation, which is consistent with the relatively higher capacity of acyl glucuronidation.

Inhibition Studies. To determine the relative importance of 2-PPA acyl glucuronidation or acyl-CoA formation on the extent of covalent binding of 2-PPA to proteins in hepatocytes, the effect of enzyme inhibitors was

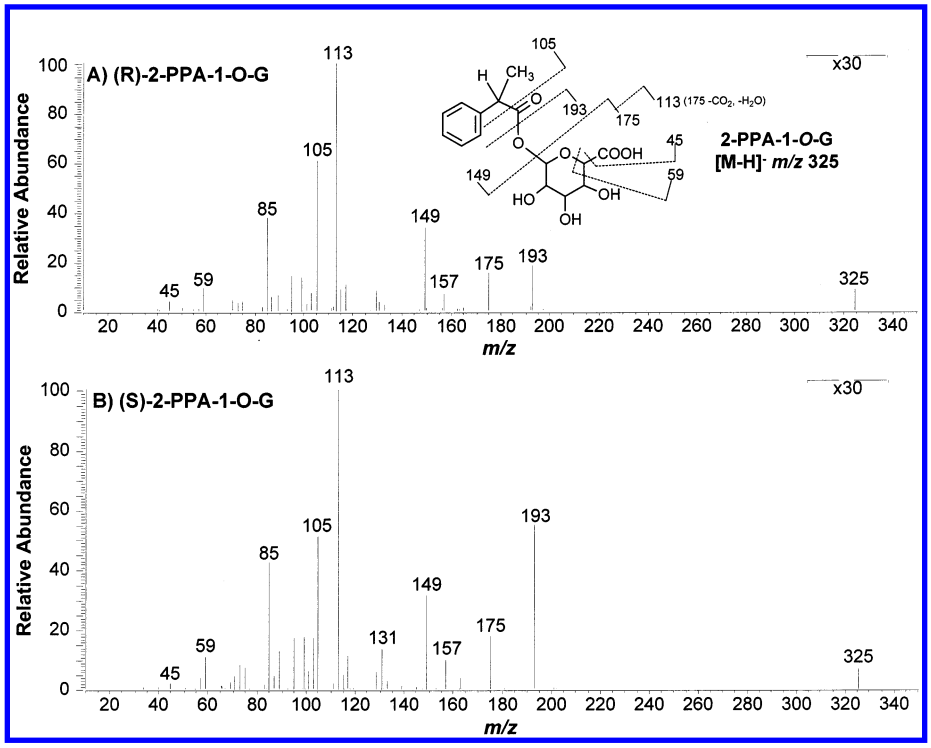

Figure 4. Spectrum of product ions formed by CID of the $[M - H]^-$ ion (*m/z* 325) of 2-PPA-1-*O*-G obtained from extracts of rat hepatocytes incubated with (A) (*R*)-2-PPA or (B) (*S*)-2-PPA.

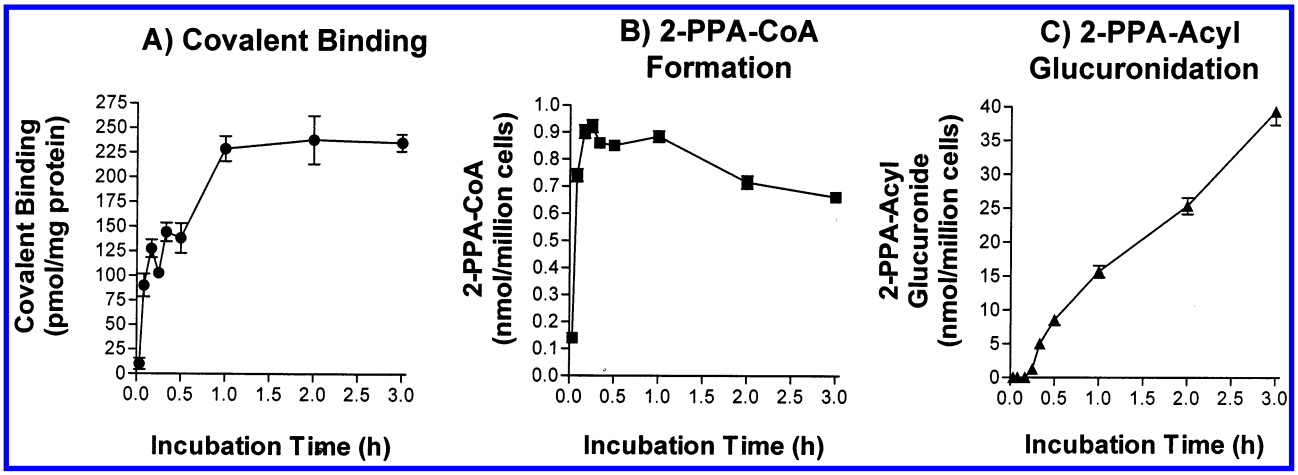

Figure 5. Time course of the (A) covalent binding, (B) acyl-CoA formation, and (C) acyl glucuronidation of (*R,S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) incubated with freshly isolated rat hepatocytes (4 million cells/mL). Values are expressed as the mean ± SD of triplicate incubations.

examined (Table 1). Inhibition experiments were performed with (*R,S*)-$^{14}C_2$-2-PPA (1 mM) incubated with hepatocytes in the presence or absence of TMA (2 mM) or (−)-borneol (1 mM) for the inhibition of 2-PPA-CoA formation and 2-PPA acyl glucuronidation, respectively. As shown in Table 1, the covalent binding of 2-PPA to hepatocyte proteins decreased by 53% in hepatocytes treated with TMA, which approximates the 66% decrease in 2-PPA-CoA formation. No significant effect by TMA was observed on 2-PPA acyl glucuronidation. Conversely, treatment with (−)-borneol, which completely inhibited the formation of 2-PPA acyl glucuronide, only decreased covalent binding by 18.7% after 3 h of incubation.

Enantioselective Studies. The formation of acyl-CoA derivatives of 2-arylpropionic acid drugs is enantioselective for the (*R*)-enantiomer, although both (*R*)- and (*S*)-isomers are known to form 1-*O*-acyl glucuronide metabolites, with enantioselectivity for the (*S*)-isomer. In the present experiments, incubations of (*R*)- or (*S*)-$^{14}C_2$-2-PPA (1 mM) with rat hepatocytes showed that this is true for 2-PPA (Figure 6). We found that the covalent binding of 2-PPA to hepatocyte proteins was 4.5-fold greater for the (*R*)-2-PPA isomer (567 pmol/mg of protein) compared to the (*S*)-2-PPA isomer (125 pmol/mg of protein) after 3 h of incubation (Figure 6A). This enantioselectivity of covalent binding correlated better with the enantioselectivity of acyl-CoA formation ($R/S = 7.0$) than with the enantioselectivity of acyl glucuronidation ($R/S = 0.67$) of the (*R*)- and (*S*)-2-PPA isomers.

Discussion

The withdrawal from the market of drugs containing a carboxylic acid moiety, especially 2-arylpropionic acid-type drugs, is disproportionally high. For these discontinued carboxylic acids, including benoxaprofen, suprofen,

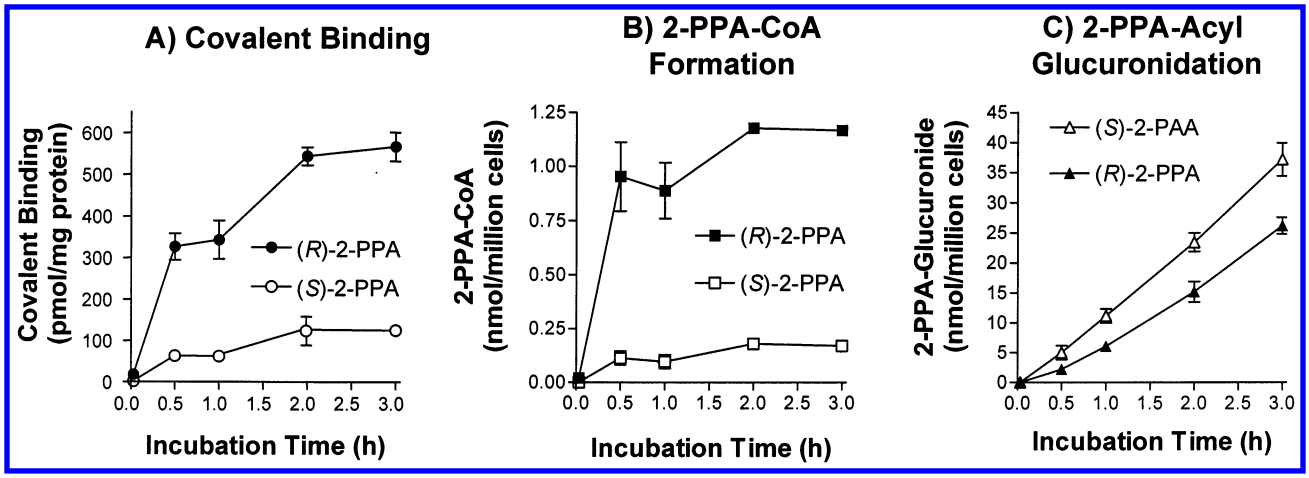

Figure 6. Time course of (A) covalent binding, (B) acyl-CoA formation, and (C) acyl glucuronidation of (*R*)-[1,2-$^{14}C_2$]-2-PPA or (*S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) incubated with freshly isolated rat hepatocytes (4 million cells/mL). Values are expressed as the mean ± SD of triplicate incubations.

Table 1. Effect of Trimethylacetic Acid (TMA) and (−)-Borneol on the Covalent Binding, Acyl-CoA Formation, and Acyl Glucuronidation of (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA in Incubations with Freshly Isolated Rat Hepatocytes[a]

	% of control		
	covalent binding	2-PPA-CoA	2-PPA glucuronide
control	100.0 ± 5.2	100.0 ± 1.2	100.0 ± 7.7
TMA-treated	47.4 ± 2.7	33.7 ± 1.4	110.0 ± 8.5
(−)-borneol-treated	81.3 ± 3.2	105.3 ± 2.5	not detected

[a] Inhibitors of metabolism, namely TMA (inhibitor of acyl-CoA formation, final concentration 2 mM) or (−)-borneol (inhibitor of glucuronidation, final concentration 1 mM), were added to hepatocyte incubations (4 million cells/mL) and a further 5 min period was allowed before the addition of (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA (final concentration 1 mM). The effects of these inhibitors on covalent binding, acyl-CoA formation, and acyl glucuronidation of 2-PPA were assessed at the 3 h time point after the addition of (*R*,*S*)-[1,2-$^{14}C_2$]-2-PPA to the incubation mixture. Control incubations were carried out without the addition of inhibitors. Values are expressed as the mean ± SD of triplicate incubations.

and pirprofen, the most frequent types of adverse reactions leading to the decision for discontinuing the products were idiosyncratic toxicities, such as liver damage, serious skin reactions and renal toxicity, sometimes associated with fever, rash, and eosinophilia (*20*, *21*). The mechanisms responsible for the initiation of such toxic side effects remain poorly understood. Covalent modification of cellular proteins by chemically reactive species formed during the metabolism of carboxylic-acid-containing drugs has been suggested as a possible mechanism to mediate some of these idiosyncratic toxicities. For acidic drugs, acyl glucuronidation and acyl-CoA thioester formation are two common metabolic pathways, both of which lead to the generation of chemically reactive metabolites (Figure 1). Covalent binding of 2-arylpropionic acid drugs to plasma proteins via their respective acyl glucuronide derivatives has been well documented during the last two decades (*2*). In contrast, few reports in the literature concern the chemical reactivity of xenobiotic acyl-CoA conjugates toward proteins. The present studies provide direct evidence, for the first time, that acyl-CoA thioesters of 2-arylpropionic-acid-type compounds can bind covalently to hepatocyte proteins, in addition to and preferentially, as compared to acyl glucuronide metabolites.

2-PPA was chosen as a simple model compound for the 2-arylpropionic acid class of drugs. The metabolism of 2-PPA has been well characterized in rats, where its major metabolite is 2-PPA acyl glucuronide and where no oxidative metabolism (phase I) has been shown (*22*). Unidirectional chiral inversion observed in vivo results from the formation of 2-PPA-CoA (*23*). Thus, 2-PPA was selected here as a model compound to investigate the relative reactivity of acyl glucuronide and acyl-CoA thioester toward hepatocyte proteins.

Time-dependent in vitro hepatocyte studies showed that covalent binding of 2-PPA to hepatocyte proteins requires prior metabolic activation of 2-PPA to acyl-CoA and acyl glucuronide derivatives (Figure 5). Covalent adduct formation was completely undetectable when both acyl-CoA formation and acyl glucuronidation were abolished in heat-denatured hepatocytes, indicating that viable cells and functional enzymes are necessary for metabolic activation of 2-PPA. Selective inhibition of each of the metabolic pathways led to a decrease in covalent adduct formation (Table 1), indicating that both metabolic pathways, acyl-CoA formation and acyl glucuronidation, are involved in the covalent binding. The extent of covalent binding of 2-PPA to hepatocyte proteins was markedly decreased by 53% when acyl-CoA formation was inhibited by 66%. By contrast, complete inhibition of acyl glucuronidation only decreased covalent adduct formation by 18.7% (Table 1). These results strongly suggest that acyl-CoA derivatives play a more important role in the covalent binding of 2-PPA to hepatocyte proteins than the respective acyl glucuronide metabolites, even though the formation of 2-PPA-acyl glucuronides (including rearranged isomers) was shown to be nearly 40-fold greater than 2-PPA-CoA formation in hepatocytes (Figure 5). The relative contribution of each metabolic activation pathway to covalent binding not only depends on the relative amount of reactive metabolite formed during the incubation, but also on the chemical reactivity of the metabolite toward proteins. A higher contribution of the acyl-CoA pathway to covalent binding therefore may result from the higher chemical reactivity of acyl-CoA thioesters toward proteins, as compared with that of acyl glucuronides. Recently, we showed, in vitro in buffer (0.05 M potassium phosphate, pH 7.4), that the reactivity of 2-PPA-SCoA with GSH (a model nucleophile) was 70 times greater than the reac-

tivity of GSH with 2-PPA-1-*O*-G, which was found to acyl-migrate to less reactive isomers (*18*).

As a model compound of 2-arylpropionic acid drugs, 2-PPA has been shown to undergo chiral inversion from the (*R*)- to the (*S*)-enantiomer via an acyl-CoA thioester intermediate (*23*). The formation of 2-PPA-CoA thioester is enantioselective for the (*R*)-enantiomer (Figure 6B). Conversely, acyl glucuronidation exhibited preference for the (*S*)-2-PPA enantiomer (Figure 6C). Both metabolic activation pathways, namely acyl-CoA formation and acyl glucuronidation, are potentially involved in covalent binding of 2-PPA to proteins in hepatocytes incubated with 2-PPA. Enantiomeric difference in the metabolic activation of (*R*)- and (*S*)-2-PPA isomers were predicted to lead to enantioselective differences in covalent binding of the acid to hepatocyte proteins. We proposed that if bioactivation by 2-PPA-CoA formation were important, then results from in vitro covalent binding studies would show increased 2-PPA-protein adduct formation in incubations with the (*R*)-2-PPA isomer. Conversely, if acyl glucuronidation was more important for covalent binding, then we predicted that the (*S*)-2-PPA isomer would form more covalent adducts. Our hepatocyte studies with (*R*)- and (*S*)-2-PPA enantiomers showed that covalent binding is enantioselective for the (*R*)-enantiomer. The extent of covalent binding was 4.5-fold greater after 3 h of incubation with (*R*)-2-PPA than with (*S*)-2-PPA (Figure 6A). Such enantioselectivity of covalent binding correlated more closely with the enantioselectivity of acyl-CoA formation ($R/S = 7.0$) than with the enantioselectivity of acyl glucuronidation ($R/S = 0.67$) of (*R*)- and (*S*)-2-PPA enantiomers. These results strongly indicate the important role of acyl-CoA thioester for 2-PPA protein adduct formation in vitro in hepatocytes, which is consistent with results obtained from inhibition studies (Table 1).

Acyl glucuronide metabolites of acidic drugs are known reactive metabolites, which readily reach the systemic circulation and are efficiently excreted into the urine and bile. Previous studies have shown that there is a direct relationship between the amount of covalent binding to plasma protein and the extent of exposure of acyl glucuronide to human plasma for each carboxylic acid drug studied in our laboratory (*24*). These studies strongly indicate the potential involvement of acyl glucuronides in the covalent binding of acidic drugs to plasma protein in vivo, which may help explain some of acidic-drugs-mediated allergic reactions (*25*). Conversely, acyl-CoA thioester derivatives, because of their large size, cannot cross plasma membranes and reach sites distant from where they are formed. It is very unlikely that acyl-CoA thioesters mediate the in vivo covalent adduct formation of acidic drugs in plasma, since acyl-CoA derivatives are not present in plasma, as opposed to the tissues where they are formed. However, it is possible that both metabolic activation pathways, namely acyl glucuronidation and acyl-CoA formation, might potentially mediate covalent binding of acidic drugs to tissue proteins and therefore contribute to drug-induced organ toxicity associated with the use of carboxylic acid drugs, since both metabolites are exposed to tissues. In vitro tissue homogenate studies with tolmetin (*11*) and diclofenac (*12*, *26*) showed that acyl glucuronides could contribute to the formation of tissue protein adducts. Recent studies with nafenopin also showed that covalent binding of nafenopin to liver proteins was directly associated with the acyl-CoA formation of nafenopin in incubations with human liver homogenate (*17*). Therefore, caution should be taken in concluding the potential involvement of metabolic activation pathways in covalent binding of acidic drugs to tissue proteins. Overemphasis of either pathway to tissue covalent binding may lead to a misinterpretation of the data. Many recent in vivo studies with carboxylic acids attributed tissue covalent adduct formation to acyl glucuronidation, for example zomepirac, diflunisal, clofibric acid, and valproic acid in drug-treated rats (*27*) and diclofenac, sulindac, and ibuprofen in drug-treated mice (*28*). However, it is well-known that clofibric acid, ibuprofen, and valproic acid are able to form acyl-CoA thioesters, which may also potentially lead to tissue covalent binding. Similarly, studies have shown that hypolipidemic agents including nafenopin, bezafibrate, and MEDICA 16 were able to acylate membrane and cytosolic liver proteins in cultured rat hepatocytes (*29*). The authors of that study attributed such selective protein binding to the formation of xenobiotic acyl-CoA thioesters. Since the authors did not have evidence that protein acylation required the prior formation of xenobiotic acyl-CoA thioesters, they were not able to eliminate the possibility of the involvement of reactive acyl glucuronide in the tissue protein covalent binding (*29*). In these cases, the potential involvement of metabolic activation by acyl-CoA formation and acyl glucuronidation in covalent adduct formation was not distinguished.

A possible explanation for the increased covalent binding in the present studies of the (*R*)-2-PPA isomer to hepatocyte protein over the (*S*)-antipode may be because of an increased ability of (*R*)-2-PPA-1-*O*-acyl glucuronide to undergo acyl migration (*18*, *30*) leading to isomers that are able to form Schiff-base-type protein adducts that rearrange to stable 1-amino-2-keto products. However, since the complete inhibition of 2-PPA acyl glucuronidation in rat hepatocyte experiments (Table 1) had only a minor effect on the covalent binding of 2-PPA to rat hepatocyte protein, we believe that acyl glucuronidation of 2-PPA has a minor effect on the enantioselectivity of covalent binding. From these studies, it is not known which type of covalent protein adducts were formed, transacylation or Schiff-base formation, from the glucuronidation pathway. In addition, studies have shown that when a racemic dose of a 2-arylpropionic acid NSAID, fenoprofen (*9*), or benoxaprofen (*31*) was given to volunteers, a slight preponderance in protein adduct concentrations in blood occurred for the (*S*)-isomers. These studies concluded that, because of stereoinversion from the (*R*)- to the (*S*)-isomer occurred, acyl glucuronide concentrations were greater for the (*S*)- than for the (*R*)-isomers leading to increased (*S*)-isomer protein adduct formation. We propose that increased tissue covalent binding of the (*S*)-2-arylpropionic acid isomer of a racemic dose will result from formation of (*R*)-2-arylpropionic acid acyl-CoA, followed by a rapid chiral inversion to the (*S*)-2-arylpropionic acid acyl-CoA derivative that then covalently binds to protein. Therefore, metabolic activation of 2-arylpropionic-acid-type drugs to acyl-CoA thioester derivatives must be considered when evaluating the enantioselectivity of irreversible binding of 2-arylpropionic acids to protein. The data presented here have changed our focus from studying reactive acyl glucuronides, which are known to covalently bind to protein via transacylation and Schiff-base-type mechanisms, to transacylation of hepatocyte proteins by reactive acyl-

CoA thioester metabolites. Furthermore, it may be useful to reexamine the studies previously carried out where only acyl glucuronides were measured, as mentioned above.

Here, we used freshly isolated rat hepatocytes to demonstrate for the first time that both metabolic pathways are involved in covalent binding of 2-PPA to protein. Importantly, the results from in vitro enzyme inhibition and enantioselective studies strongly indicate that the 2-PPA-acyl-CoA thioester metabolite is more important that 2-PPA acyl glucuronide in the covalent binding of this model 2-arylpropionic acid to hepatocyte protein. Finally, from the results presented here, we propose that metabolic activation of 2-arylpropionic acid drugs by acyl-CoA formation will lead to protein covalent binding and therefore could contribute to the potential tissue toxicity of this class of drugs.

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