COVALENT BINDING OF 2-PHENYLPROPIONYL-S-ACYL-COA THIOESTER TO TISSUE PROTEINS IN VITRO

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ABSTRACT:

In this study, we investigated the possible involvement of acyl-CoA, reactive intermediary metabolites of 2-arylpropionic acids (profens), in protein adduct formation in rat liver homogenate and in human serum albumin (HSA) in buffer. (RS)-[1-14C]-2-Phenylpropionic acid ([14C]-2-PPA, 1 mM) was incubated with rat liver homogenate (1.5 mg/ml) in the presence of cofactors of acyl-CoA formation (Mg2+, ATP, and CoA). Aliquots of the incubation mixture were analyzed for covalent binding and acyl-CoA formation over a 3-h period. High-performance liquid chromatographic analysis of the products from such incubations showed the presence of 2-phenylpropionyl-S-acyl-CoA (2-PPA-CoA), which was confirmed by coelution with authentic 2-PPA-CoA, as well as by mass spectrometry. In the same incubations, 2-PPA was shown to bind covalently to hepatic proteins in a time- and ATP-dependent fashion. Inhibition of 2-PPA-CoA formation by acyl-CoA synthetase inhibitors, such as palmitic acid, lauric acid, octanoic acid, and ibuprofen, markedly decreased the extent of covalent binding of 2-PPA to hepatic proteins. Results from these in vitro studies strongly suggest that acyl-CoA thioester derivatives are chemically reactive and are able to bind covalently to tissue proteins in vitro, and, therefore, may contribute significantly to covalent adduction formation of profen drugs in vivo.

2-Arylpropionic acids (profens) are a commonly used class of nonsteroidal anti-inflammatory drugs, widely prescribed as analgesic, antipyretic, and anti-inflammatory agents. Conjugation with glucuronic acid is a major route for the biotransformation and elimination of profen drugs, such as ibuprofen, carprofen, fenoprofen, and naproxen (Spahn-Langguth et al., 1997). Studies have shown that these acyl-linked glucuronides are chemically reactive species that undergo hydrolysis to regenerate the pharmacologically active parent drug and that also undergo intramolecular acyl migration to yield β-glucuronidase-resistant isomers (Spahn-Langguth and Benet, 1992; Spahn-Langguth et al., 1997; Li and Benet, 2002). More importantly, these electrophilic metabolites have been shown to bind covalently to serum albumins in vitro and to plasma and tissue proteins in vivo (Spahn-Langguth and Benet, 1992; Li and Benet, 2002); and, therefore, metabolic activation of profen drugs by acyl glucuronidation has been proposed as a metabolic route to mediate the idiosyncratic liver toxicity associated with the use of profen drugs (Boelsterli et al., 1995).

Another metabolic route of profen drugs is the acyl-CoA thioester pathway, recently recognized also as yielding reactive metabolites of acidic drugs. 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-CoAs, including palmitoyl-CoA and arachidonoyl-CoA, can react nonenzymatically with sulfhydryl groups on proteins and peptides in vitro in a time- and concentration-dependent fashion (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). Our recent studies with 2-phenylpropionic acid (2-PPA) demonstrated that 2-phenylpropionyl-S-acyl-CoA (2-PPA-CoA) was able to acylate glutathione sulfhydryl to form 2-PPA-S-acyl-glutathione at a rate that was approximately 70-fold more rapid than the similar reactions with 2-PPA-1-O-acyl glucuronides (Li et al., 2002). The present study was designed to examine the covalent binding of 2-PPA-CoA to human serum albumin and rat liver homogenate in vitro. 2-PPA was chosen because it is the simplest congener of profen drugs and is capable of forming 2-PPA-CoA, as inferred from the unidirectional chiral inversion of 2-PPA in vivo (Fournel and Caldwell, 1986). Results from these in vitro studies strongly suggest that acyl-CoA thioesters of profen drugs are chemically reactive electrophiles and are able to bind covalently to tissue proteins in vitro, which may, therefore, contribute significantly to covalent adduction formation of profen drugs in vivo, in addition to acyl glucuronides.

Materials and Methods

Materials. (R)-(-)-2-PPA, perchloric acid (70%), and EDTA were purchased from Aldrich Chemical Co. (Milwaukee, WI). CoA, ATP, MgCl2.

Abbreviations used are: 2-PPA, 2-phenylpropionic acid; 2-PPA-CoA, 2-phenylpropionyl-S-acyl-CoA; DTT, dithiothreitol; HSA, human serum albumin; HPLC, high-performance liquid chromatography.

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dithiothreitol (DTT). Triton X-100, human serum albumin (HSA; ≥96% albumin, essentially fatty acid-free), palmitic acid, octanoic acid, lauric acid, ibuprofen, Tris-HCl, trimethylamine, and potassium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). (RS)-[1-14C]-2-PPA-CoA was synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO). (RS)-[1-14C]-2-PPA-CoA and authentic (R)-2-PPA-CoA were synthesized by conventional procedures employing chloroformate, as we reported previously (Li et al., 2002).

**Incubation of (RS)-[1-14C]-2-PPA-CoA with HSA.** Fatty acid-free HSA (30 mg/ml) was incubated with (RS)-[1-14C]-2-PPA-CoA (0.1 Ci/mmol, 0.1 mM) or (RS)-[1-14C]-2-PPA (0.1 Ci/mmol, 0.1 mM, negative control) in 0.05 M potassium phosphate buffer (pH 7.4) at 37°C, in triplicate. After 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h of incubation, aliquots (1 ml) of the reaction mixture were taken and added to a solution of perchloric acid (7%, 0.5 ml), vortex mixed, and centrifuged (1500g) for 10 min. The supernatants were neutralized by 1 N NaOH and analyzed by reverse-phase HPLC for the stability of 2-PPA-CoA thioester. Covalent binding of 2-PPA to HSA was measured by scintillation counting of exhaustively washed protein pellets, as described previously (Li et al., 2002).

**Animals and Tissue Collection.** Male Sprague-Dawley rats (~240–260 g; B&K Universal, Fremont, CA) were anesthetized with ether, the abdominal cavities were opened, and the livers were perfused with ice-cold saline. Perfused livers were rapidly removed and frozen immediately in liquid nitrogen. Tissue homogenates were prepared from livers of six rats as reported (Tracy et al., 1993).

**2-PPA-CoA Formation in Rat Liver Homogenate.** Incubations of (R)-2-PPA (0–4 mM) with rat liver homogenate (0.25–1.0 mg/ml) were carried out in triplicate in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM MgCl₂, and 2.5 mM ATP in a final volume of 0.5 ml. After a 3-min preincubation, reactions were initiated by the addition of ATP and allowed to proceed for 30 min at 37°C, at which time the incubations were stopped by the addition of 50 µl of perchloric acid (7%). After centrifugation at 10,000g for 5 min, the supernatants were neutralized with 1 N NaOH and analyzed by reverse-phase HPLC. The above incubation conditions were established to be linear with respect to both time and protein concentration; the cofactors were present at saturating concentrations and the concentrations of MgCl₂, and Triton X-100 were optimal for 2-PPA-CoA formation. The presence of 2 mM EDTA and 1 mM DTT showed no significant effect on 2-PPA-CoA formation during 30-min incubations. Values for Kᵣ and Vᵣmax were determined by an extended least-squares regression modeling program (WinNonlin software; Pharsight, Mountain View, CA).

**Covalent Binding Studies with Rat Liver Homogenate.** Incubations were carried out in triplicate at 37°C in 150 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM (RS)-[1-14C]-2-PPA-CoA (2 Ci/mmol, 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM MgCl₂, 2.5 mM ATP, and 1.5 mg/ml rat liver homogenate. Samples were preincubated at 37°C for 3 min, and the reactions were initiated by the addition of ATP. Control incubations were conducted without the addition of ATP. Aliquots (2 ml) of the incubation mixture were taken at indicated times and quenched with 20 µl of 70% perchloric acid for determination of acyl-CoA and covalent adduct formation. After centrifugation at 10,000g for 5 min, the supernatants were neutralized with 1 N NaOH and analyzed by reverse-phase HPLC for the formation of 2-PPA-CoA thioester. Covalent binding of 2-PPA to proteins was measured by scintillation counting of exhaustively washed hepatic protein precipitates as described by Sallustio et al. (2000).

**Inhibition of 14C-2-PPA-CoA Formation and Covalent Binding.** Inhibition studies were conducted with the following four acids: palmitic acid (0.1 mM), lauric acid (0.2 mM), octanoic acid (2 mM), and ibuprofen (1 mM), which are known to form acyl-CoA thioesters themselves. The concentrations of inhibitors were chosen to be saturating with respect to their acyl-CoA thioester formation (Knights and Jones, 1992; Tracy et al., 1993). Preliminary studies showed that the four acids significantly inhibited 2-PPA-CoA formation by more than 40% at the concentrations chosen. Samples were incubated for 3 h using a standard reaction mixture as described above for covalent binding studies with rat liver homogenate, except that the inhibitors dissolved in ethanol were added and then dried under nitrogen before addition of the remaining incubation components. All incubation series included negative controls lacking ATP.

**HPLC Analysis.** The formation of 2-PPA-CoA in rat liver homogenates was analyzed on an SB-C₈ Zorbax column (150 × 4.6 mm; MAC-MOD Analytical, Chadds Ford, PA) at a flow rate of 1.0 ml/min. The isocratic running buffer containing 17.5% acetonitrile in 0.19 M ammonium acetate buffer (pH 7.0) was used with UV detection at 262 nm. Standard curves for 2-PPA-CoA in rat tissue homogenates and HSA were prepared by spiking synthetic (R)-2-PPA-CoA standards to rat tissue homogenates according to the procedures described above.

**Protein Assay.** Protein concentrations were measured by the Pierce BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard.

**Results and Discussion.** Covalent binding of (RS)-[1-14C]-2-PPA-CoA to HSA (a model protein) in phosphate buffer at pH 7.4 and 37°C was shown to be time-dependent. Approximately 970 pmol/mg HSA covalent adduct
was detected after 24 h of incubation (Fig. 1A). The hydrolysis of 2-PPA-CoA to 2-PPA free acid was accelerated in the presence of HSA. The disappearance of 2-PPA-CoA thioester in HSA buffer solution followed first-order kinetics with an apparent half-life of 13.4 h (Fig. 1B). However, in the absence of HSA, 2-PPA-CoA is very stable, showing negligible hydrolysis even after 24 h of incubation. The apparent half-life of 2-PPA-CoA in phosphate buffer (pH 7.4, 37°C) is approximately 12 days (Li et al., 2002). These findings are consistent with current concepts that albumin possesses some esterase activity, as described previously with acyl glucuronides, such as zomepirac and oxaprozin (Smith et al., 1985; Wells et al., 1987).

The extent of covalent binding of 2-PPA-CoA to serum albumin seemed to depend upon the species source, with human albumin forming more adduct than bovine. Consistent with the covalent binding profiles, the accelerated hydrolysis of 2-PPA-CoA was not observed with bovine serum albumin during 24 h of incubations (data not shown), but was seen with HSA. Similar observations were also reported for acyl glucuronides. Tolmetin and zomepirac acyl glucuronides (Munafo et al., 1990; Smith et al., 1990) were shown to exhibit much less covalent binding with bovine serum albumin as compared with HSA. Watt and Dickinson (1990) also reported that covalent binding of diflunisal glucuronide was greater with fatty acid-free HSA than with rat serum albumin and human plasma proteins. These findings suggest that small differences in albumin, such as animal origin and purity, can be important for the stability and covalent binding of chemically reactive species, including acyl-CoA.

**FIG. 2.** Time-dependent 2-PPA-CoA formation (A) and covalent binding of 2-PPA to hepatic proteins (B) in rat liver homogenate incubations with (RS)-[1-14C]-2-PPA (1 mM) carried out at 37°C in triplicate in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM MgCl2, 2.5 mM ATP, and 1.5 mg/ml rat liver homogenate. Control incubations were conducted without the addition of ATP. Values represent means ± S.D. from triplicate incubations.

**FIG. 3.** Effect of various acyl-CoA synthetase inhibitors on 2-PPA-CoA formation (A) and covalent binding of 2-PPA to hepatic proteins (B). Triplicate incubations were carried out for 3 h at 37°C in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM MgCl2, 2.5 mM ATP, 1 mM (RS)-[1-14C]-2-PPA, and 1.5 mg/ml rat liver homogenate in the presence and absence of various inhibitors. The rat liver homogenate was preincubated with the inhibitor for 3 min. Blank incubations were conducted without the addition of ATP. Values represent means ± S.D. from triplicate incubations.
thioesters and acyl glucuronides. Caution should be taken when comparing covalent binding data from various sources. Nonetheless, regardless of the nature of the albumin preparation used, 2-PPA-CoA was shown to be reactive toward serum albumin in vitro, suggesting its potential to bind covalently to other tissue proteins as well.

HPLC analysis of tissue homogenate incubations with (R)-2-PPA in the presence of cofactors for acyl-CoA formation showed the presence of 2-PPA-CoA, which was confirmed by coelution with authentic 2-PPA-CoA and by mass spectrometry. 2-PPA-CoA was undetectable when ATP was absent (Fig. 2A). Lineweaver-Burk plots of the data from rat liver homogenate clearly demonstrate that more than one enzyme was involved in the catalysis of 2-PPA-CoA formation. The apparent $K_m$ and $V_{max}$ for the low-affinity and high-capacity enzyme were 1.9 ± 0.5 mM and 351 ± 44 pmol/min/mg protein (mean ± S.D.; n = 3), respectively. There were clear indications of a high-affinity, low-capacity enzyme with an apparent $K_m$ and $V_{max}$ approximating 4.1 ± 0.2 μM and 16.8 ± 0.6 pmol/min/mg protein. This conclusion was also supported by inhibition studies. Both palmitic acid (long-chain fatty acid) and octanoic acid (medium-chain fatty acid) were shown to efficiently inhibit 2-PPA-CoA formation, suggesting the possible involvement of both medium- and long-chain fatty acyl-CoA synthetase (Fig. 3A). Preliminary studies showed that propionic acid (2 mM) had little effect on 2-PPA-CoA formation, suggesting the possible involvement of both medium- and long-chain fatty acyl-CoA synthetase in this process.

Covalent binding of 2-PPA to hepatic proteins via the acyl-CoA pathway was examined in similar incubations. Our results demonstrate that protein covalent binding appears to depend on the formation of 2-PPA-CoA (Fig. 3). Both processes were time- and ATP-dependent. When (RS)-[1-$^{14}$C]-2-PPA was incubated with rat liver homogenate and cofactors for acyl-CoA formation (CoA, ATP, and Mg$^{2+}$), 2-PPA-CoA formation and covalent binding were both detected. In the absence of ATP, no 2-PPA-CoA was formed and little covalent binding was observed (Fig. 2). When the formation of 2-PPA-CoA was inhibited, the extent of covalent binding decreased (Fig. 3). For most inhibitors, both processes decreased proportionally, so that the ratio of covalent binding to 2-PPA-CoA formation remained unchanged (Table 1). This finding suggests that the 2-PPA-CoA derivative could be an important reactive metabolite, mediating covalent binding to hepatic proteins.

The studies described here convince us that covalent binding of xenobiotic carboxylic acids to hepatic proteins can be mediated by the acyl-CoA thioester pathway. However, caution should be taken in concluding that the acyl-CoA formation pathway is the major pathway for covalent binding of profen drugs. Two alternative metabolic activation pathways of profen drugs, namely, acyl glucuronidation and acyl-CoA formation, can lead to the covalent adduct formation in tissues. Our subsequent publications will examine the comparison of the two pathways following in vivo 2-PPA dosing.

### TABLE 1

<table>
<thead>
<tr>
<th>Covalent Binding to 2-PPA-CoA Forma4tion</th>
<th>Covalent Binding</th>
<th>Covalent Binding to 2-PPA-CoA Ratio</th>
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<tbody>
<tr>
<td>Control$^a$</td>
<td>3.92 ± 0.67</td>
<td>1.72 ± 0.12</td>
</tr>
<tr>
<td>Palmitic acid$^b$</td>
<td>2.34 ± 0.26</td>
<td>1.09 ± 0.14</td>
</tr>
<tr>
<td>Ibuprofen$^b$</td>
<td>1.90 ± 0.07</td>
<td>1.07 ± 0.18</td>
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<tr>
<td>Lactic acid$^b$</td>
<td>1.32 ± 0.09</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td>Octanoic acid$^b$</td>
<td>0.38 ± 0.02</td>
<td>0.57 ± 0.05</td>
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</table>

$^a$ Control incubations were carried out for 3 h at 37°C in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM MgCl$_2$, 2.5 mM K$_2$HPO$_4$, 1 mM ATP, and 1.5 mg/ml rat liver homogenate.

$^b$ The rat liver homogenate was preincubated with the inhibitor for 3 min. The concentrations for the inhibitors used in the incubations are as follows: 0.1 mM palmitic acid, 1 mM ibuprofen, 0.2 mM lactic acid, and 2 mM octanoic acid.

$^c$ $P < 0.05$ compared to corresponding control group, using t test.

$^d$ $P < 0.01$ compared to corresponding control group, using t test.

Acknowledgment. We thank Milagros Hann for assistance in performing HPLC analyses.

### References


Li ET al. Effect of various acyl-CoA synthetase inhibitors on 2-PPA-CoA formation, covalent binding of 2-PPA to hepatic proteins, and the ratio of covalent binding to 2-PPA-CoA formation.

Results are shown as means ± S.D. of triplicate incubations in rat liver homogenate.