Reactive Phase II Metabolites of Carboxylic Acid Drugs: Acyl-CoA Derivatives *versus* Acyl Glucuronides

by

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DISSERTATION

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To my parents and

my beloved husband, Yan

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Chunze Li

December 2002

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Abstract

Reactive Phase II Metabolites of Carboxylic Acid Drugs: Acyl-CoA *versus* Acyl Glucuronide Pathways

Chunze Li

Increasing evidence suggests that many carboxylic acid containing drugs are metabolized to reactive intermediates that can form irreversible adducts with proteins. Such adducts have been proposed to mediate the idiosyncratic toxicity associated with the use of acidic drugs. Early studies in our laboratory concentrated on acyl glucuronide metabolites. However, it is now recognized that many acidic drugs are metabolized to a second reactive electrophilic metabolite, namely acyl-CoA thioesters. Here we describe studies primarily with a model carboxylic acid, 2-phenylpropionic acid (2-PPA), to characterize and compare these two metabolic activation pathways and quantitate each pathway's contribution to protein covalent adduct formation in vitro and in vivo in rats. Studies with glutathione (GSH) showed that both metabolic activation pathways were able to acylate the GSH sulfhydryl group to form the 2-PPA-*S*-acyl glutathione (2-PPA-SG) conjugate. The reactivity of 2-PPA-*S*-acyl CoA with GSH was 70 times greater than that of GSH with glucuronide. The total formation of 2-PPA-SG in vivo in rats, as indicated by the total amount of 2-PPA-SG-derived products excreted in urine and bile, was enantioselective for the (*R*)-isomer (*R/S* = 3.3), which correlated more closely with the enantioselectivity of acyl-CoA formation (*R/S* = 7.5) than with that of acyl glucuronidation (*R/S* = 0.6) of (*R*)- and (*S*)-2-PPA enantiomers. Inhibition experiments with 2-PPA in rat hepatocytes and in vivo in rats showed that inhibition of acyl-CoA

formation by trimethylacetic acid, but not acyl glucuronidation by (-)-borneol, led to marked decreases in covalent binding to protein. Conversely, increase of acyl-CoA formation in clofibric acid-treated or diabetic rats led to a significant increase in 2-PPA covalent adduct formation. Enantioselective studies with (*R*)- and (*S*)-2-PPA in freshly isolated rat hepatocytes showed that covalent binding to protein was enantioselective for the (*R*)-isomer (*R/S* = 4.5), which correlated well with the enantioselectivity of acyl-CoA formation (*R/S* = 7.0), but not with acyl glucuronidation (*R/S* = 0.67). Together, these results suggest that acyl-CoA thioesters of carboxylic acid drugs are reactive acylating species that potentially can contribute to protein covalent binding in addition to the respective acyl glucuronides.

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Introduction

1.1 Background

Xenobiotic carboxylic acids are a commonly encountered class of compounds, widely used as non-steroidal anti-inflammatory drugs (NSAIDs, e.g., ibuprofen and diclofenac), diuretic agents (e.g., furosemide and ethacrynic acid), an antiepileptic drug (valproic acid), and hypolipidemic agents (fibric acid derivatives, e.g., clofibric acid and gemfibrozil; HMG-CoA reductase inhibitors, e.g., simvastatin and cerivastatin). Although xenobiotic carboxylic acids are generally well tolerated, in rare cases the use of carboxylic acid drugs has been associated with the development of severe and sometimes fatal idiosyncratic toxicity. It is striking that of 47 drugs withdrawn from the U.S., British, and Spanish markets from 1964 through 1993 due to severe toxicity, 10 are carboxylic acids (Bakke et al., 1984; Bakke et al., 1995). For all these discontinued carboxylic acids (alclofenac, bendazac, benoxaprofen, fenclofenac, ibufenac, indoprofen, pirprofen, suprofen, ticrynafen and zomepirac) 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 (Bakke et al., 1984; Zimmerman, 1990;

Zimmerman, 1994; Bakke et al., 1995). Recently, cerivastatin, an HMG-CoA reductase inhibitor, has also been withdrawn from the market due to a high risk for severe muscle toxicity (SoRelle, 2001; Thompson, 2001).

Although the overall incidence of such idiosyncratic toxicity for acidic drugs is low, the outcome can be severe, sometimes fatal. The idiosyncratic toxicities associated with the use of carboxylic acid drugs are of major concern clinically because they can not be predicted from preclinical safety studies and also because they are largely independent of the dose administered and host-dependent (Boelsterli et al., 1995). Although the mechanisms underlying acidic drug-induced idiosyncratic toxicity are still poorly understood, covalent modification of tissue proteins by reactive metabolites of acidic drugs is often suggested as a possible mechanism (Boelsterli et al., 1995; Pumford and Halmes, 1997).

1.2 Acyl Glucuronides

Acyl glucuronidation is a major route for the biotransformation and elimination of carboxylic acid drugs (Spahn-Langguth and Benet, 1992; Li and Benet, 2002). These acyl-linked glucuronides have been shown to be reactive electrophilic metabolites, capable of undergoing hydrolysis to regenerate the pharmacological active parent drug and undergoing intramolecular rearrangement to yield β-glucuronidase resistant isomers (Compernolle et al., 1978; Spahn-Langguth and Benet, 1992; Li and Benet, 2002). More importantly, these electrophilic metabolites can bind covalently to serum albumin in vitro and to plasma and tissue protein in vivo (Etter-Kjelsaas and Kuenzle, 1975; Spahn-Langguth et al., 1997; Li and Benet, 2002).

1.2.1 Biochemical Aspects of Acyl Glucuronides

The formation of acyl glucuronides is catalyzed by membrane-bound enzymes, uridine 5′-diphosphate (UDP)-glucuronosyltransferases (UGTs, EC 2.4.1.17), which transfer the glucuronic acid from UDP-glucuronic acid (UDPGA) to the carboxyl group of the aglycone, resulting in ester-linked glucuronides. The mechanism of the reaction catalyzed by UGTs is an S_N2-type reaction. The anomeric center undergoes inversion during the enzymatic transfer of α-D-glucuronic acid in UDPGA to the acceptor substrate, resulting in the formation of the β-configuration. UGTs are a family of closely related isoenzymes mainly located in the endoplasmic reticulum and exhibiting different, but overlapping, substrate specificities (Mackenzie et al., 1997; Ritter, 2000). Several rat and human UGTs have been identified. Human UGTs 1A3, 1A9, and 2B7 and rat 2B1 have been shown to catalyze the acyl glucuronidation of many carboxylic acid drugs, including NSAIDs (e.g., diclofenac (King et al., 2001), ibuprofen, ketoprofen, naproxen, pirprofen, flurbiprofen, diflunisal, mefenamic acid), an antiepleptic drug (valproic acid), diuretic agents (e.g., furosemide) and fibric acid derivatives (e.g., clofibric acid, bezafibrate, ciprofibrate) (Ritter, 2000). Human UGTs 1A1 and 1A3 were shown to catalyze the acyl glucuronidation of HMG-CoA reductase inhibitors (also called "statins", e.g., simvastatin, lovastatin, and cerivastatin) (Prueksaritanont et al., 2002).

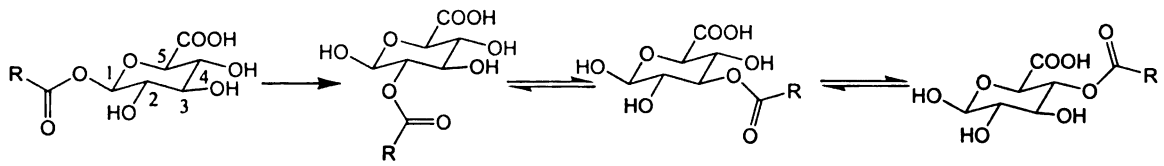
1.2.2 Hydrolysis and Acyl Migration

Hydrolysis of an acyl glucuronide leads to regeneration of the pharmacologically active parent drug. Potential catalysts include hydroxide ion, β-glucuronidases, serum albumin, and esterases. Rates of hydrolysis are dependent on pH and temperature, with

more rapid degradation of the enzymatically formed β-1-*O*-acyl glucuronide at physiological and higher pH than under more acidic conditions. The rate of chemical hydrolysis decreases significantly in cold and acidic conditions (pH 3-4), but hydrolysis may still occur slowly during freezing and especially during thawing (Upton et al., 1982). This may result in a substantial increase in the concentration of the parent compound and may be responsible for some of the variation in the apparent extent of the amount excreted unchanged in the urine of some drugs as reported by different investigators. Acyl glucuronides can undergo substantial hydrolysis to the parent aglycone in vivo and this may be due to enzymatic cleavage by β-glucuronidase or nonspecific esterases under physiological conditions. Degradation of the conjugates in bile and in the intestines will contribute to the enterohepatic recirculation of the parent compound.

Intramolecular acyl migration is a well-established reaction in carbohydrate chemistry (Haine, 1976) and is mechanistically related to alkaline hydrolysis. Migration of the acyl moiety occurs from the 1-carbon hydroxyl group to the neighboring 2-, 3-, and 4-hydroxyl groups of the glucuronyl moiety (Figure 1.1). This results in the formation of β-glucuronidase-resistant glucuronic acid esters that exhibit chromatographic properties different from the β-1-*O*-acyl glucuronide. Intramolecular acyl migration was first demonstrated for bilirubin glucuronide. Studies with endogenous bilirubin-IXα-glucuronides collected from bile demonstrated a sequential migration of the original biosynthetic 1-*O*-acyl glucuronide to 2-, 3-, and 4-*O*-isomers (Compernolle et al., 1977; Compernolle et al., 1978). Subsequently, studies of acyl glucuronides of various xenobiotic carboxylic acids have shown intramolecular acyl migration to be a general

phenomenon for acyl glucuronides (Spahn-Langguth and Benet, 1992; Li and Benet, 2002).



1-*O*-acyl glucuronide 2-*O*-acyl isomer 3-*O*-acyl isomer 4-*O*-acyl isomer

Figure 1.1 Migration of the acyl group of the β-1-*O*-acyl glucuronide from C1 to C2, C3, and C4 of the glucuronic acid ring. The rearrangement is reversible with one exception: the C1-isomer is not formed from the C2-isomer.

The mechanism of acyl migration is well established and proceeds via nucleophilic attack on the neighboring hydroxyl group and formation of an ortho-ester intermediate (Bradow et al., 1989; Haine, 1976). In situ mechanistic studies with ^{1}H-NMR spectroscopy of HPLC-purified isomers have determined the order of migration to be from the biosynthetic glucuronide to the 2-*O*-isomer followed by formation of the 3- and 4-*O*-isomers. Migration between the three positional isomers is reversible but reformation of the parent 1-*O*-β-acyl glucuronide is very unlikely owing to the mutarotation at C-1 after movement of the acyl group (Figure 1.1). The studies of Bradow et al. (1989) indicated that there is no evidence for rearrangements beyond nearest-neighbor hydroxyl groups.

The degradation of 1-*O*-acyl glucuronides (including hydrolysis and acyl migration) follows apparent first-order kinetics over the measurable concentration range. Stability studies with acyl glucuronides of a number of carboxylic acids demonstrate that

intramolecular acyl migration under physiological conditions is the predominating reaction in the early stages of the in vitro incubations, whereas hydrolysis of 1-*O*-acyl glucuronide and its isomers becomes the more important reaction at later times or under alkali conditions (Hasegawa et al., 1982).

The rates of acyl migration and hydrolysis vary among different compounds and are influenced by many factors. As detailed studies for zomepirac glucuronide (Hasegawa et al., 1982; Smith et al., 1985a) and tolmetin glucuronide (Munafo et al., 1990) demonstrate, the rates of acyl migration and hydrolysis depend on the pH, temperature, and composition of the medium. Acyl glucuronides are most stable at pH 2-4, low temperatures, and in the presence of esterase inhibitors.

Stability of acyl glucuronides is also affected by the presence of protein. Ruelius et al. (1986) found accelerated degradation of oxaprozin glucuronide in human serum albumin (HSA) and plasma. Indeed, they showed that albumin was catalytic for all three reactions (intramolecular acyl migration, hydrolysis, and covalent binding). Reports in the literature suggest that the effects of albumin or plasma on the stability of acyl glucuronide conjugates and their isomers vary with the drugs studied. HSA has been shown to enhance the degradation rates of acyl glucuronides of many carboxylic acid-containing drugs, including zomepirac (Smith et al., 1985a), oxaprozin (Ruelius et al., 1986; Wells et al., 1987), fenoprofen (Volland et al., 1991), etodolac (Smith et al., 1992), ketoprofen (Hayball et al., 1992), naproxen (Bischer et al., 1995), clofibric acid (Grubb et al., 1993), and gemfibrozil (Sallustio et al., 1997). An opposite (stabilizing) effect of HSA was observed for tolmetin glucuronide, but bovine serum albumin (BSA) cause an increase in the rate of hydrolysis (Munafo et al., 1990). In the presence of HSA,

degradation of diflunisal (Watt and Dickinson, 1990), salicylic acid (Dickinson et al., 1994), mefenamic acid (McGurk et al., 1996), and furosemide (Mizuma et al., 1999) glucuronides in albumin solution was retarded in comparison to that found in buffer alone, while no significant change of the degradation rate of ibufenac glucuronide was observed with or without HSA (Castillo and Smith, 1995).

1.2.3 Covalent Binding of Acyl Glucuronides to Proteins

Covalent binding of acyl glucuronide to proteins was first described for bilirubin (Etter-Kjelsaas and Kuenzle, 1975; McDonagh et al., 1984). Subsequently, acyl glucuronides of many carboxylic acid-containing drugs have been shown to bind covalently to plasma proteins, especially albumin in vitro, including nonsteroidal anti-inflammatory drugs [benoxaprofen (van Breemen and Fenselau, 1985), indomethacin (van Breemen and Fenselau, 1985), flufenamic acid (van Breemen and Fenselau, 1985), oxaprozin (Ruelius et al., 1986), zomepirac (Smith et al., 1986), tolmetin (Munafo et al., 1990), carprofen (Iwakawa et al., 1988), fenoprofen (Volland et al., 1991), naproxen (Bischer et al., 1995), diflunisal (Watt and Dickinson, 1990), salicylic acid (Dickinson et al., 1994), etodolac (Smith et al., 1992), suprofen (Smith and Liu, 1993), ibuprofen (Castillo and Smith, 1995), ibufenac (Castillo and Smith, 1995), ketoprofen (Dubois et al., 1993), and mefenamic acid (McGurk et al., 1996)], the uricosuric drug probenecid (Hansen-Moller and Schmit, 1991), the hypolipoproteinemic reagents [clofibric acid (Grubb et al., 1993; van Breemen and Fenselau, 1985), fenofibric acid (Grubb et al., 1993), gemfibrozil (Sallustio et al., 1997), and beclobric acid (Mayer et al., 1993)], the

diuretic agent furosemide (Mizuma et al., 1999), and the antiepileptic drug valproic acid (Williams et al., 1992). The in vivo formation of covalently bound plasma protein adducts by acyl glucuronides has been demonstrated in humans for a number of acidic drugs, including beclobric acid (Mayer et al., 1993), clofibric acid (Sallustio et al., 1991), carprofen (Iwakawa et al., 1988), diclofenac (57), diflunisal (McKinnon and Dickinson, 1989), fenoprofen (Volland et al., 1991), gemfibrozil (Sallustio and Foster, 1995), ketoprofen (Dubois et al., 1993), probenecid (McKinnon and Dickinson, 1989), salicylic acid (Dickinson et al., 1994), tolmetin (Munafo et al., 1993; Zia-Amirhosseini et al., 1994), valproic acid (Williams et al., 1992), and zomepirac (Smith et al., 1986).

The in vitro covalent binding of acyl glucuronides was shown to be dependent on time (van Breemen and Fenselau, 1985; Wells et al., 1987), pH (Smith et al., 1986; Munafo et al., 1990; Smith and Liu, 1993), glucuronide concentration (Dubois et al., 1993), and origin of albumin (Munafo et al., 1990; Watt and Dickinson, 1990). For oxaprozin glucuronide (Ruelius et al., 1986; Wells et al., 1987), the highest yield of protein adduct was obtained after the glucuronide and HSA were incubated at pH 7 for approximately 1 h at 37°C. Similarly, maximum covalent binding to HSA for zomepirac glucuronide occurred after 1 h incubation at pH 9, although the level of protein adducts decreased rapidly after this time owing to the instability of the adducts at this pH. High concentrations of adduct were also observed after 6 h incubation of zomepirac glucuronide and HSA at pH 7 and 8 at 37°C (Smith et al., 1986). The in vitro covalent binding of suprofen glucuronide to HSA was shown to increase with increasing pH at 37°C and to be time-dependent (Smith and Liu, 1993). The extent of covalent binding of ketoprofen glucuronide (Dubois et al., 1993) to albumin was proportional to acyl

glucuronide concentration over the range studied (from 11.62 to 69.72 μM). Watt and Dickinson (1990) showed that covalent binding of diflunisal glucuronide is greater with fatty-acid-free HSA than with rat serum albumin (RSA) and human and rat plasma proteins, and suggested that the different animal origins and the state of purity of albumin might be important for the stability and covalent binding of acyl glucuronides. Similar findings were also reported for tolmetin glucuronide (Munafo et al., 1990). The extent of covalent binding of tolmetin glucuronide with BSA was much less than, but the rate of adduct formation was the same as, that with HSA.

In addition to 1-*O*-acyl glucuronide, the isomeric conjugates could also form covalent protein adducts. Isomeric conjugates of zomepirac glucuronide (Smith et al., 1986; Smith et al., 1990) were found to covalently bind to HSA, at somewhat decreased extents as compared to the β-1-*O*-acyl glucuronide itself (% bound: C1> C2> C4> C3). Reports in the literature suggest that certain isomeric conjugates were even more reactive toward proteins than the β-1-*O*-acyl glucuronide. Isomers of suprofen glucuronide exhibited time-dependent covalent binding and this binding was 38% higher than that of the β-1-*O*-acyl glucuronide (Smith and Liu, 1993). Similarly, protein adduct formation of valproic acid (Williams et al., 1992), salicylic acid (Dickinson et al., 1994), etodolac (Smith et al., 1992), and diflunisal (Dickinson and King, 1991) was shown to be much more rapid and extensive from isomeric glucuronide conjugates than from the β-1-*O*-acyl glucuronides. However, not all of the isomeric conjugates are important for covalent binding. Ruelius et al. (1986) reported that only the β-1-*O*-acyl glucuronide of oxaprozin, not the isomers, led to significant irreversible binding.

1.2.4 Mechanisms of Covalent Binding of Acyl Glucuronides to Proteins

Two mechanisms have been proposed to describe the irreversible binding of acyl glucuronides to proteins (Smith et al., 1990). The first is a nucleophilic displacement mechanism whereby a protein nucleophile (-NH, O, S) attacks the carbonyl group of the aglycone (Figure 1.2). In this mechanism, a bond (amide, ester, or thioester) is formed between protein and the drug, and glucuronic acid is liberated. The second mechanism of covalent binding of acyl glucuronides to proteins is analogous to the nonenzymatic glycosylation of albumin (Garlick and Mazer, 1983) and requires prior acyl migration of the drug moiety away from the biosynthetic β-1-*O*-acyl glucuronide to permit ring opening of the sugar. The reactive aldehyde group so exposed can then reversibly form an imine (Schiff's base) with an amine group on protein. Subsequent Amadori rearrangement has been proposed to yield a stable ketoamine derivative (Figure 1.2). In contrast to the nucleophilic displacement, both drug and glucuronic acid moieties (still linked together by an ester group) become bonded to proteins.

1.2.5 Predictability of the Covalent Binding of Acidic Drugs

The accumulated data from a number of studies suggest that the extent of covalent binding for carboxylic acid drugs in vitro may be predicted on the basis of the degradation rate constant (including hydrolysis and acyl migration) of the glucuronide conjugate. An analysis of published data (Benet et al., 1993) on the covalent binding of several acyl glucuronides indicates that there is a good linear correlation between the

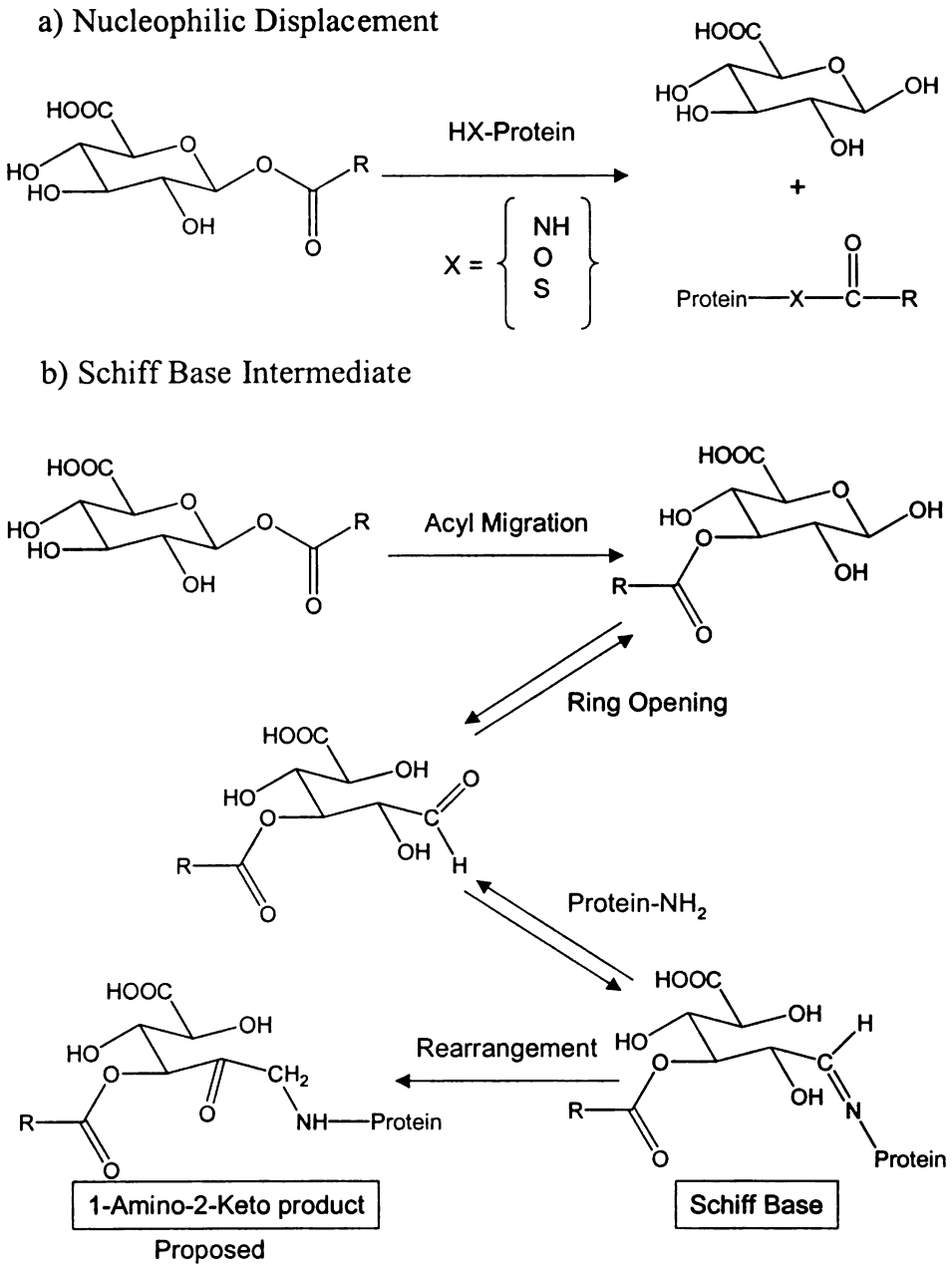


Figure 1.2 Proposed mechanisms for covalent binding of acyl glucuronides to proteins: a) Nucleophilic displacement mechanism, b) Schiff base intermediate mechanism.

apparent first-order disappearance rate constant for an acyl glucuronide in buffer, which is a measure of its chemical reactivity, and the maximum covalent binding observed when the glucuronide is incubated with HSA in vitro (Figure 1.3). Acyl glucuronides of arylacetic acid (α-unsubstituted) such as tolmetin and zomepirac exhibit the highest covalent binding and lowest stability (highest degradation rate). The intermediate stable glucuronides of 2-arylpropionic acids (mono α-substituted), such as carprofen and fenoprofen, have lower covalent binding. Lowest covalent binding is observed for the most stable fully substituted carboxylic acids, such as beclobric acid and furosemide. Figure 1.3 summarizes data from our laboratories over a 6-year period with respect to in vitro degradation rates of the β-1-*O*-acyl glucuronides and the in vitro covalent binding for nine drug molecules, suggesting that the extent of in vitro covalent binding to albumin is predictable based on the chemical structure of the acid and depends on the degree of substitution at the alpha carbon to the carboxylic acid.

As expected from the in vitro covalent binding, the degree of in vivo covalent binding to plasma proteins should depend, at least, on the plasma concentrations of the acyl glucuronides and the degradation rate of each conjugate. The plasma concentrations of acyl glucuronides vary with the drug studied and are dependent on the rate of formation, degradation and elimination, as well as the administered dose. Acyl glucuronides of some carboxylic acids may reach significant concentrations in plasma of humans, as shown for zomepirac (Smith et al., 1985b), tolmetin (Hyneck et al., 1988), diflunisal (McKinnon and Dickinson, 1989), beclobric acid (Mayer et al., 1993), and etodolac (Smith et al., 1992), while no oxaprozin (Ruelius et al., 1986) and fenofibric

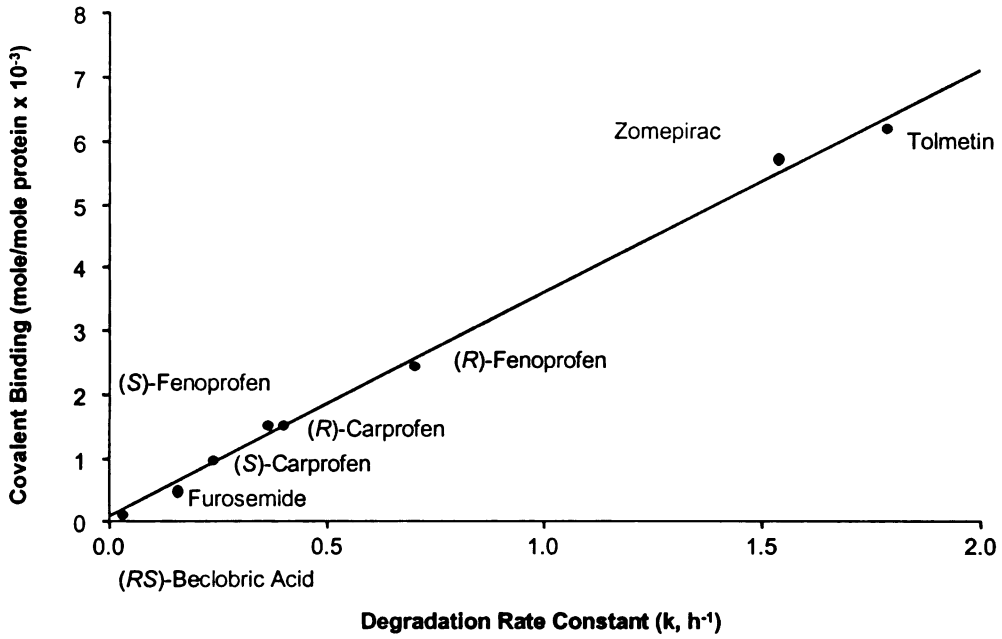


Figure 1.3 Plot of maximum epitope density (moles drug covalently bound per mole of protein x 10^{-3}) versus degradation rate constant (h^{-1}) for the in vitro incubation of various acyl glucuronides (1 μM) in the presence of human serum albumin (0.5 mM). Degradation rates reflect both acyl migration and hydrolysis. Results are obtained from seven different studies over a six-year period utilizing purified β-1-*O*-acyl glucuronides of zomepirac (Smith et al., 1986), tolmetin (Munafo et al., 1990), carprofen (Iwakawa et al., 1988), fenoprofen (Volland et al., 1991), furosemide (Sekikawa et al., 1995; Mizuma et al., 1999), and beclobric acid (Mayer et al., 1993). The data points for (+) and (-) enantiomers of beclobric acid are indistinguishable on the scale used (Benet et al., 1993).

acid (Wel et al., 1988) glucuronides have been detected in human plasma. In vivo studies with five carboxylic acid drugs, at their usual therapeutic doses, in five different sets of healthy volunteers, showed a 30-fold variation in AUCs for acyl glucuronides, whereas the maximum plasma protein binding showed a 25-fold variation (Table 1.1). Since for each drug there is a direct relationship between the amount of covalent binding and the extent of exposure of acyl glucuronide (AUC), we normalized bound drug to AUC for comparison with in vitro glucuronide degradation rates, yielding a highly significant linear correlation ($r^2 = 0.873$). The findings presented in Table 1.1 suggest that the in vivo covalent binding of acidic drugs to albumin in humans is also predictable on the basis of the degradation rate constant of the glucuronide conjugate when the extent of covalent binding is corrected for the levels of the glucuronide present in plasma (AUC).

Table 1.1 In vivo bound drug, area under the plasma drug glucuronide concentration time curve (AUC) and in vitro acyl glucuronide degradation rates*

Parent Compound	Bound drug (mole/mole protein) x 10^4	AUC Glucuronide (mole x h/L) x 10^6	Bound/AUC (mole drug x L/(mole protein x mole glucuronide x h)) x10^{-2}	k h^{-1}
tolmetin	2.77 ±1.54	3.72 ± 0.95	0.75	1.78
zomepirac	2.33 ± 0.45	6.41 ± 2.14	0.36	1.54
(*R*)-fenoprofen	1.02 ± 0.32	6.31 ± 5.65	0.16	0.71
(*S*)-fenoprofen	3.23 ± 0.85	60.4 ± 24.7	0.054	0.36
racemic carprofen	1.92 ± 1.28	40.9 ± 7.3	0.047	0.32
(+)-beclobric acid	0.12 ± 0.03	8.16 ± 1.34	0.015	0.031
(-)-beclobric acid	0.20 ± 0.11	8.31 ± 1.63	0.024	0.027

* Measurement of maximum amount of drug covalently bound to human serum albumin and area under the plasma concentration time curve (AUC) for the glucuronide conjugates measured in five different groups of healthy volunteers following oral dosing of either 400 mg of tolmetin (Hyneck et al., 1988), 100 mg of zomepirac (Smith et al., 1986), 600 mg of racemic fenoprofen (Volland et al., 1991), 50 mg of racemic carprofen (Iwakawa et al., 1988), or 100 mg of racemic beclobric acid (Mayer et al., 1993). When covalently bound drug is normalized to area under the curve for the respective glucuronide conjugates, an excellent correlation with the in vitro degradation rate constant (k) is obtained with an r^2 of 0.873.

1.3 *Acyl-Coenzyme A Thioesters (Acyl-CoA)*

It has been recognized for many years that enzymatic activation of endogenous fatty acids to their corresponding high energy coenzyme A (CoA) thioesters is required for their use in fatty acid synthesis, β-oxidation, triacylglycerol formation, protein acylation and other intracellular processes. Like endogenous fatty acids, xenobiotic carboxylic acids also can form acyl-CoA thioesters. Formation of acyl-CoA thioesters is critically important in the biochemistry of xenobiotic carboxylic acids and in intermediary metabolism. The activated acyl-CoA thioester may serve as an obligatory intermediate for the formation of amino acid conjugates (Hutt and Caldwell, 1990), acyl carnitine (Knights, 1998) and choline derivatives (Sastry et al., 1997) (Figure 1.4).

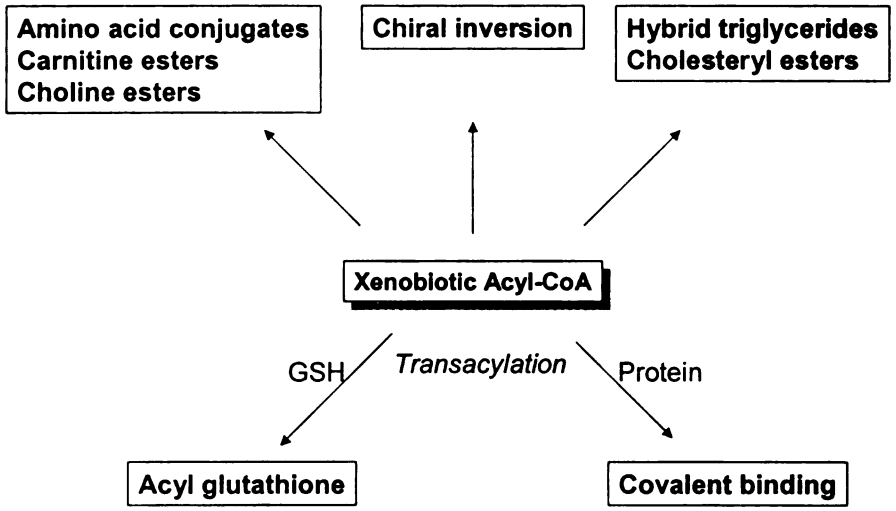
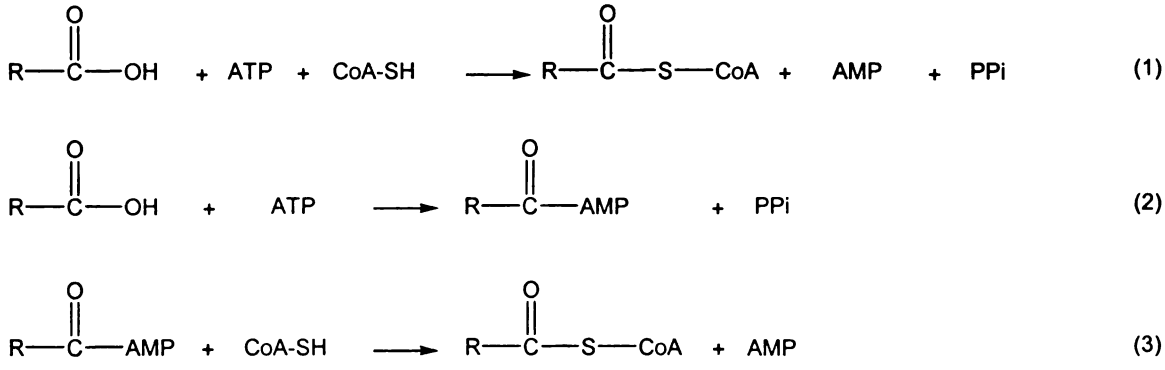


Figure 1.4 Potential routes of metabolism of xenobiotic acyl-CoA thioesters.

Xenobiotic acyl-CoA derivatives could also participate in lipid metabolism pathways resulting in the formation of hybrid triacylglycerides and cholesteryl esters (Fears, 1985) (Figure 1.4). In the case of the 2-arylpropionic acid NSAIDs, the acyl-CoA thioesters undergo inversion of configuration of the 2-propionic acid moiety, which results in the formation in vivo of the pharmacological active (*S*)-enantiomers from the inactive (*R*)-isomers (Hutt and Caldwell, 1983; Caldwell et al., 1988). Therefore, it is significant that formation of acyl-CoA thioesters appears to be a key step for many of these transformations (Figure 1.4).

1.3.1 Biochemical Aspects of Acyl-CoA formation

The formation of acyl-CoA thioesters is generally catalyzed by acyl-CoA synthetase (ACS, EC 6.2.1.1-2.1.3). The overall reaction catalyzed by ACS is shown in reaction (1). It is generally believed that the reaction proceeds via a ping-pong mechanism in which the first step is an adenylyl transfer forming an acyl-adenylate and pyrophosphate (PPi, reaction 2) (Watkins, 1997). The activated intermediate, which was shown to be enzyme-bound, is then attacked by CoA-SH, yielding the acyl-CoA product and AMP (reaction 3). Since the nucleotidyl transfer is thermodynamically near



equilibrium, the reaction is generally "pulled" by the action of inorganic Pyrophosphatases, the enzymes that hydrolyze PPi to inorganic phosphate (Watkins, 1997).

The ACS enzymes have been classified based on their ability to conjugate fatty acids with different carbon chain lengths, namely short-chain ACS (C_2-C_4), medium-chain ACS (C_4-C_{12}), long-chain ACS (C_{10}-C_{22}) and very long-chain ACS (>C_{22}) (Knights, 1998; Knights and Drogemuller, 2000). The enzymes are located in various cell compartments, e.g., cytosol, smooth endoplasmic reticulum, mitochondria and peroxisomes (Table 1.2). Tissue distribution of the enzymes is widespread, with highest activity associated with liver and adipose tissue.

Table 1.2 Fatty acid acyl-CoA synthetase (ACS)

	Chain length specificity	Subcellular localization	Xenobiotic substrates
Short-chain ACS	C_2-C_4	Cytosol, mitochondrial matrix	
Medium-chain ACS	C_4-C_{12}	Mitochondrial matrix	Arylacetic and aromatic carboxylic acids, 2,4-D, IB, VPA, TMA[a]
Long-chain ACS	C_{10}-C_{22}	Microsomes, peroxisomes, mitochondrial membrane	2-Arylpropionic acids, "fibric acids"
Very long-chain ACS	> C_{22}	Microsomes, peroxisomes	

[a]Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetic acid; IB, ibuprofen; VPA, valproic acid; TMA, trimethylacetic acid.

In addition to their role in fatty acid metabolism, the medium and long-chain ACS have been implicated in the metabolism of a variety of xenobiotic carboxylic acids. Many arylacetic and aromatic carboxylic acids, including phenylacetic acid, 1-naphthylacetic acid, benzoic acid and salicylic acid, have been shown to be substrates of medium-chain ACS, which is located in the mitochondrial matrix (Table 1.2). Other compounds known to be mitochondrial toxins, such as 2,4-dichlophenoxyacetic acid (2,4-D), ibuprofen (IB), trimethylacetic acid (TMA) and valproic acid (VPA), were also shown to be substrates for medium-chain ACS (Table 1.2). The preferred substrates for long-chain ACS include a variety of hypolipidaemic and peroxisome-proliferating agents (e.g., clofibric acid, ciprofibric acid, nafenopin and phthalate plasticizers) and 2-arylpropionic acid non-steroidal anti-inflammatory drugs (e.g., ibuprofen, fenoprofen, ketoprofen and benoxaprofen) (Table 1.2).

1.3.2 Chemical Reactivity of Acyl-CoA Thioesters

Acyl-CoA thioesters of xenobiotic and endogenous carboxylic acids have a reactive potential due to the presence of the relatively electrophilic thioester carbonyl carbon. For example, the free amine of glycine, even under conditions where the amine is protonated (pH 7.5), is acylated by salicyl-CoA to form salicyluric acid (Tishler and Goldman, 1970). A number of studies have been conducted in order to characterize the non-enzymatic acylation of protein sulfhydryls by endogenous acyl-CoA derivatives in vitro (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). In such experiments, endogenous acyl-CoA derivatives, including palmitoyl-CoA and arachidonoyl-CoA, have been shown to react spontaneously with cysteine-containing proteins and peptides to

form thioester-conjugates in a time- and concentration-dependent fashion. Since xenobiotic acyl-CoA derivatives possess the same reactive thioester-linked carbonyl carbon, we propose that similar reactions to proteins nucleophiles may occur in vivo with potentially reactive xenobiotic acyl-CoA derivatives formed during the metabolism of xenobiotic carboxylic acids (Hertz and Bar-Tana, 1988; Sallustio et al., 2000).

1.4 Objective and Overview of Thesis

The overall aim of this thesis is to investigate the chemical reactivity of acyl-CoA thioesters of xenobiotic carboxylic acids and compare their reactivity with that of their respective acyl glucuronides both in vitro and in vivo. We speculate that xenobiotic acyl-CoA derivatives are reactive acylating reagents that may contribute to the covalent binding of carboxylic acids to proteins and that could be responsible for some of the idiosyncratic toxicity associated with the use of acidic drugs. Here we describe studies primarily with the model carboxylic acid, 2-phenylpropionic acid (2-PPA), designed to characterize and compare the two metabolic activation pathways and to quantitate each pathway's contribution to protein covalent adduct formation in rat hepatocytes and in vivo in rats. Specific aims of the research were:

1. **To determine the relative ability of acyl glucuronide and acyl-CoA thioester metabolites of 2-PPA to transacylate the nucleophilic cysteinyl-thiol of glutathione (GSH) in vitro in buffer.** GSH, a cytoprotective tripeptide with a nucleophilic cysteinyl thiol, is able to react with electrophiles spontaneously and serves as a model nucleophile for comparing the electrophilicity of reactive metabolites of xenobiotics. Presumably, the greater the reactivity of the acylating

metabolite with GSH in vitro, the higher the probability that the metabolite will acylate protein nucleophiles in vivo, and the greater the risk of toxicity. This work is discussed in detail in Chapter 2.

2. **To investigate the chemical reactivity of the acyl-CoA thioester of 2-PPA towards protein nucleophiles such as human serum albumin and rat hepatic proteins.** 2-PPA-1-*O*-acyl glucuronide (2-PPA-1-*O*-G) has been shown to be chemically reactive and able to bind covalently to serum albumin in vitro (Georges et al., 1999; Akira et al., 2000). However, there is no information on the chemical reactivity of 2-PPA-*S*-acyl Coenzyme A (2-PPA-CoA) with proteins. Chapter 3 characterizes the chemical reactivity of 2-PPA-CoA thioester and examines its possible involvement in protein adduct formation in rat liver homogenate and human serum albumin (HSA) in buffer.
3. **To quantitate the relative contribution of acyl-CoA formation and acyl glucuronidation of 2-PPA to protein covalent adduct formation in vitro in rat hepatocytes and in vivo in rats.** Chapters 4 and 5 examine the relative contribution of each metabolic activation pathway to covalent adduct formation in freshly isolated hepatocytes (Chapter 4) and in vivo in rats (Chapter 5), using two independent approaches — inhibition of 2-PPA acyl-CoA formation and acyl glucuronidation and their effect on covalent binding of ^{14}C-labeled 2-PPA to rat hepatic proteins (Chapters 4 and 5), and enantioselective studies with ^{14}C-labeled (*R*)- and (*S*)-2-PPA in rat hepatocytes (Chapter 4).
4. **To evaluate the inductive effects of clofibric acid on 2-PPA acyl-CoA formation, acyl glucuronidation and the extent of covalent binding of 2-PPA to rat tissue**

proteins. Clofibric acid, a hypolipidemic agent, is known to be a potent peroxisomal proliferator in rats (Hawkins et al., 1987). Clofibric acid induces several hepatic enzymes associated with fatty acid metabolism, including acyl-CoA synthetases, the enzymes that catalyze the formation of acyl-CoA thioesters (Schoonjans et al., 1993; Alegret et al., 1994). Therefore, clofibric acid treatment could modulate the extent of covalent binding of 2-PPA by increasing the exposure of 2-PPA-CoA thioester to liver proteins. This possibility is investigated in Chapter 6.

5. **To examine the effects of diabetes (type I) on metabolic activation of 2-PPA in vitro in hepatocytes.** Diabetes mellitus is known to lead to profound changes in whole body fatty acid metabolism. Uncontrolled diabetes leads to a fatty acid overload, which is accompanied by an increased capacity for oxidation of fatty acid by both mitochondrial and peroxisomal liver pathways (Mannaerts and Van Veldhoven, 1992). The activation of endogenous fatty acids to their corresponding high-energy acyl-CoA thioesters is required for their cellular usage as in β-oxidation in mitochondria and peroxisomes. Diabetes markedly increases the activity of acyl-CoA synthetase, the enzyme responsible for the formation of acyl-CoA thioester (Asayama et al., 1999). A significant increase of the hepatic concentration of free CoA, a cofactor that is essential for acyl-CoA formation, was observed in diabetic rats (Horie et al., 1986). Therefore, we propose that diabetes might increase the extent of covalent binding of acidic drugs to proteins by increasing the exposure of their acyl-CoA thioesters to tissues. The objective of chapter 7 is to examine the effect of type I diabetes on the metabolic activation of 2-PPA in hepatocytes as a first step to

understanding if diabetic patients are at an increased risk for the idiosyncratic toxicity associated with the use of acidic drugs.

6. **To assess the enantioselective acylation of glutathione by (*R*)- and (*S*)-2-PPA metabolites in vivo in rats.** GSH is a model nucleophile for comparing the electrophilicity of reactive metabolites of xenobiotics. The relative ability to acylate glutathione in vivo may be proportional to the relative ability to acylate protein nucleophiles in vivo, which may lead to toxic side effects associated with the use of carboxylic acid-containing drugs (Boelsterli et al., 1995). By using an enantioselective approach, we attempted to identify the major metabolic activation pathway of 2-PPA that leads to the formation of acyl glutathione in vivo in rats. This work is presented in Chapter 8.
7. **To characterize the chemical reactivity of *S*-acyl-CoA thioesters of 2,4-dichlorophenoxyacetic acid (2,4-D), phenylacetic acid (PAA) and 2-PPA with biological nucleophiles, such as GSH and HSA.** Structure activity studies have shown that the degree of the substitution at the α-carbon of carboxylic acids can be used to predict the extent to which their respective 1-*O*-acyl glucuronides undergo hydrolysis, acyl migration and covalent binding to protein in vitro and in vivo (Benet et al., 1993). Similar structure activity relationships should exist for the ability of *S*-acyl-CoA metabolites to acylate biological nucleophiles. In Chapter 9 we test this possibility by using three carboxylic acids with different substitutions at the α-carbon.

Chapter 2

Studies on the Chemical Reactivity of 2-Phenylpropionic Acid 1-*O*-Acyl Glucuronide and *S*-Acyl-CoA Thioester Metabolites[1]

2.1 Introduction

Many carboxylic acid-containing drugs have been shown to covalently bind to protein in exposed patients (Smith et al., 1986; Benet et al., 1993; Zia-Amirhosseini et al., 1994). One type of covalent modification occurs when acidic drugs become irreversibly bound to protein *via* acyl-linkages with protein nucleophiles (Ding et al., 1995; Qiu et al., 1998a). Two alternative mechanisms for the acylation of protein by reactive metabolites of carboxylic acid-containing drugs, as shown in Figure 2.1, include reaction of protein nucleophiles with chemically unstable acyl glucuronides (Faed, 1984; Smith et al., 1986; Li and Benet, 2002) or with chemically reactive acyl-CoA thioesters derivatives (Tishler and Goldman, 1970; Sallustio et al., 2000; Grillo and Benet, 2002). Many acidic drugs are metabolized to both acyl glucuronide and acyl-CoA derivatives in vivo, e.g., many 2-

[1]This chapter was published as a manuscript entitled "Studies on the chemical reactivity of 2-phenylpropionic acid 1-*O*-acyl glucuronide and *S*-acyl-CoA thioester metabolites" Li C, Grillo MP, Benet LZ, *Chem Res Toxicol* **15**: 1309-1317, 2002. Reproduced with permission.

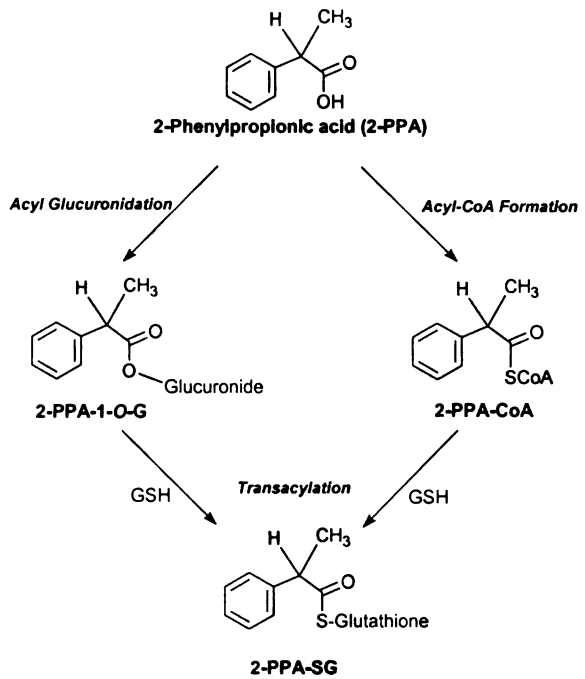


Figure 2.1 Proposed scheme for the metabolic activation of 2-phenylpropionic acid by acyl glucuronidation and acyl-CoA formation followed by reaction with glutathione.

arylpropionic acid nonsteroidal anti-inflammatory drugs (NSAIDs) (Caldwell et al., 1988), lipid lowering agents (Lygre et al., 1986), and the antiepileptic drug valproic acid (Baillie, 1992). Recently, we showed that the acyl-CoA thioester derivative of the lipid lowering drug clofibric acid was able to acylate glutathione (GSH) sulfhydryl to form clofibryl-*S*-acyl-glutathione (Grillo and Benet, 2002) in a fashion that was nearly 40-fold more rapid than similar experiments conducted with clofibryl-1-*O*-acyl glucuronide (Shore et al., 1995). These glutathione conjugation reactions may also be occurring in vivo since the *S*-acyl mercapturic acid conjugates, as well as the *S*-acyl-glutathione derivatives, have been detected in the urine of clofibrate-treated patients (Stogniew and Fenselau, 1982) and in the bile of clofibric acid-dosed rats, respectively (Shore et al.,

1995). Recently, we detected another *S*-acyl-glutathione degradation product, namely clofibryl-*N*-acyl-cysteine in the urine of clofibrate-dosed rats (Grillo and Benet, 2001). Such *N*-acyl-cysteine derivatives are formed from a *S* to *N* rearrangement reaction that occurs at the γ-glutamyltranspeptidase step in degradation of *S*-acyl-glutathione conjugates towards the formation of mercapturic acid derivatives (Tate, 1975).

In the present work, an attempt was made to determine which of these acylating derivatives may be more important in the transacylation of protein nucleophiles. Acyl-CoA thioesters, because of their high-energy carbon-sulfur bond (Huxtable, 1986), have been shown recently to be reactive acylating derivatives with proteins. Such studies included the covalent binding of nafenopin-CoA to proteins in vitro in incubations with human hepatic proteins plus the cofactors for acyl-CoA formation (CoA, ATP, Mg^{2+}) (Sallustio et al., 2000), the time-dependent acylation of proteins and peptides by palmitoyl-CoA (Bharadwaj and Bizzozero, 1995), and the non-enzymatic acylation of glycine by salicyl-CoA in vitro (Tishler and Goldman, 1970). Here, we performed experiments to determine the relative abilities of the acyl-CoA thioester and 1-*O*-acyl glucuronide derivatives of 2-phenylpropionic acid (2-PPA) to acylate the cysteinyl-thiol of GSH. 2-PPA, the simplest congener representing the 2-aryl-propionic acid class of NSAIDs that have been shown to covalently bind to protein (Spahn-Langguth and Benet, 1992; Li and Benet, 2002) and cause idiosyncratic drug reactions in patients (Boelsterli et al., 1995), was used in these experiments because it is known to be metabolized by both acyl glucuronidation (Meffin et al., 1986) and acyl-CoA formation (Fournel and Caldwell, 1986). These studies were designed to obtain information on the relative chemical reactivities of 2-PPA acyl glucuronide and 2-PPA-acyl-CoA derivatives that

will support future studies to determine the relative importance of acyl glucuronidation and acyl-CoA formation on the covalent binding of 2-PPA to protein in vitro in rat hepatocytes and in vivo in rats. More specifically, experiments were designed to evaluate the reactivity differences between 2-PPA-1-*O*-acyl glucuronide (2-PPA-1-*O*-G) and 2-PPA-*S*-acyl-CoA thioester (2-PPA-CoA) with glutathione, in that the relative ability to acylate glutathione in vitro may be proportional to the relative ability to acylate protein nucleophiles in vivo, which may lead to toxic side effects associated with the use of carboxylic acid-containing drugs (Boelsterli et al., 1995). The present studies include experiments with pure (*R*)- and (*S*)-2-PPA anomers of the 1-*O*-acyl glucuronides and acyl-CoA thioesters to see if the stereochemistry of 2-PPA affects the chemical reactivity of the derivative. In addition, the ability of 2-PPA-CoA to acylate bovine serum albumin BSA was determined to evaluate its ability to acylate nucleophiles other than the cysteinyl-sulfhydryl of GSH.

2.2 Experimental Section

2.2.1 Chemicals

2-PPA, (*R*)-(-)-2-PPA, (*S*)-(+)-2-PPA, GSH, fatty acid free BSA, *S*-carboxymethyl-BSA, and CoA were purchased from Sigma Chemical Co. (St. Louis, MO). Triethylamine, ethyl chloroformate, monobasic potassium phosphate, potassium bicarbonate, THF (anhydrous), iodoacetic acid, sodium acetate, and hydroxylamine were purchased from the Aldrich Chemical Co. (Milwaukee, WI). [1,2-$^{14}C_2$]-phenylacetate was purchased from Moravek Biochemicals (Brea, CA). 2-PPA-CoA, 2-PPA-SG, [1,2-

$^{14}C_2$]-2-PPA, [1,2-$^{14}C_2$]-2-PPA-CoA and 2-PPA-1-*O*-G were synthesized as described below. All solvents used for HPLC were of chromatographic grade.

2.2.2 Instrumentation and Analytical Methods

HPLC was carried out on a Shimadzu LC-600 isocratic system coupled to a Shimadzu SPD-6AV UV-Vis detector. Electrospray LC-MS and LC-MS/MS of synthetic standards and in vitro biological extracts was performed on a Hewlett Packard HP 1100 LC/MSD bench-top electrospray mass spectrometer and a Finnigan-MAT TSQ 7000 (San Jose, CA), respectively. ^{1}H NMR spectra were recorded on a General Electric QE300 spectrometer operating at 300 MHz. Chemical shifts are reported in parts per million (δ) downfield from the internal standard, tetramethylsilane.

2.2.3 Biosynthesis of (*R*)-2-PPA- and (*S*)-2-PPA-1-O-Acyl Glucuronides

Male Sprague-Dawley rats (220 g) were given doses of (*R*)-2-PPA or (*S*)-2-PPA (200 mg/kg, ip), dissolved in distilled water (1.0 mL) and neutralized with NaOH (1N, 1 equivalent). Sixteen hours postadministration, during which time the animals (one rat for each enantiomer) were kept in metabolic cages unrestrained, total urines (~20 mL) were collected over 2 mL of 1N HCl and extracted with ethyl acetate (1 x 20 mL), dried ($MgSO_4$), and evaporated to dryness with N_2 gas at room temperature. Residues were dissolved in a solution of 50% acetonitrile in ammonium acetate (10 mM, pH 5.0) and analyzed by reverse phase isocratic HPLC on a Microsorb-MV C_{18} column (150 x 4.6 mm, 5μ, Rainin LC and Supplies, Walnut Creek, CA) at a flow rate of 1.8 mL/min. The

mobile phase consisted of 0.1% TFA in 15% acetonitrile; 226 nm was the wavelength used for UV detection. Treatment of urine extracts with β-glucuronidase at pH 5.0 and room temperature followed by HPLC analysis at 0 and 30 min revealed the peak corresponding to β-1-*O*-acyl glucuronides (10.8 min for (*R*)- and 11.8 min for (*S*)-2-PPA-1-*O*-acyl glucuronide), which were absent in the 30 min β-glucuronidase treated extracts. The 2-PPA formed upon incubation with β-glucuronidase eluted on HPLC (as above) at 23.8 min for both enantiomers. The (*R*)- and (*S*)-2-PPA-1-*O*-acyl glucuronides were purified by HPLC and shown to be enantiomerically pure by chiral HPLC analysis of the 2-PPA obtained after β-glucuronidase treatment of the purified 1-*O*-acyl glucuronides. Chiral HPLC was performed on a chiral column (Chiralcel OJ-R, 150 x 4.6 mm, Chiral Technologies Inc., Exton, PA) at a flow rate of 0.8 mL/min with UV detection at 226 nm. Mass spectrometric analysis of the purified 2-PPA-1-*O*-acyl-glucuronides by LC/MS was performed in the negative ion mode with a fragmentor voltage of 130 that provided mass spectra consistent with their structures: (*R*)-2-PPA-1-*O*-G *m/z* 325 ($[M-H]^-$, 30%), *m/z* 193 ([glucuronic acid]$^-$, 56%), *m/z* 175 ($[C_6H_7O_6]^-$, 68%), *m/z* 149 ($[C_9H_9O_2]^-$, 61%), *m/z* 113, 100%. (*S*)-2-PPA-1-*O*-G *m/z* 325 ($[M-H]^-$, 18%), *m/z* 193 ([glucuronic acid]$^-$, 100%), *m/z* 175 ($[C_6H_7O_6]^-$, 41%), *m/z* 149 ($[C_9H_9O_2]^-$, 49%), *m/z* 113, 72%.

2.2.4 Synthesis of 2-PPA-SG Thioester

The synthesis and purification of the *S*-acyl-glutathione thioester of 2-PPA was performed by conventional procedures employing ethyl chloroformate (Stadtman, 1957), exactly as we previously reported for the synthesis and purification of clofibryl-*S*-acyl-

glutathione (Grillo and Benet, 2001). LC-MS/MS analysis was performed by gradient elution from 5% to 100% acetonitrile over 20 min in 0.1% TFA on a Zorbax C18 reverse-phase column (150 x 2.1 mm, 5μ, MAC-MOD Analytical, Chadds Ford, PA) at a flow rate of 0.3 mL/min. 2-PPA-SG eluted at a retention time of 6.95 min and gave a tandem mass spectrum consistent with its structure (Baillie and Davis, 1993) (CID of MH^+ ion at *m/z* 440), *m/z* (%): *m/z* 365 ($[M + H - Gly]^+$, 8%), *m/z* 293 ([M + H – pyroglutamic acid]$^+$, 54%), *m/z* 236 ($[C_6H_5\text{-}CH(CH_3)COSCH_2CH{=}NH_2]^+$, 45%), *m/z* 208 ($[C_6H_5\text{-}CH(CH_3)COSCH_2CH(NH_2)(C{=}O)]^+$, 100%), *m/z* 130 ([pyroglutamic acid]$^+$, 12%), *m/z* 105 ([C6H5-CH(CH3)]$^+$, 14%), *m/z* 76 ([glycine]$^+$, 12%). ^1H NMR ($^2H_6$-DMSO): δ 1.41-1.43 (d, 3H, -CH_3), 1.81 (m, 1H, Glu-β), 1.89 (m, 1H, Glu-β'), 2.26 (m, 2H, Glu-γ,γ'), 2.91 (m, 2H, Cys-β,β'), 3.28 (m, 2H, J = 6.4 Hz, Glu-α), 3.67 (m, 2H, Gly-α,α'), 3.99-4.01 (q, 1H, -CH-), 4.37 (m, 1H, Cys-α), 7.25-7.36 (m, 5H, phenyl ring), 8.42-8.44 (d, 1H, Cys NH), 8.70 (br, 1H, Gly NH).

2.2.5 Synthesis of [1,2-$^{14}C_2$]-2-PPA

The synthesis of (*RS*)-[1,2-$^{14}C_2$]-2-PPA from methyl [1,2-$^{14}C_2$]-phenylacetate ($C_6H_5{}^{14}CH_2{}^{14}CO_2CH_3$) was carried out as follows: Briefly, 0.74 mL of a solution (obtained from the supplier) containing 0.74 mCi of [1,2-$^{14}C_2$]-phenylacetic acid (100 mCi/mmol, 1 mCi/mL of methanol) was added to phenylacetic acid (1g, 7.4 mmol) to give [1,2-$^{14}C_2$]-phenylacetic acid (1 g, 7.4 mmol, 0.1 mCi/mmol), all of which then was added to a solution of methanolic-HCl (30 mL) and stirred overnight at room temperature. The methanol then was removed by evaporation under reduced pressure

followed by dissolving the residue in ethyl ether (100 mL) and drying with anhydrous magnesium sulfate. The magnesium sulfate was removed by filtration and the ether filtrate evaporated under reduced pressure, which provided a clear colorless oil of methyl [1,2-$^{14}C_2$]-phenylacetate (~1 g, 91% yield). The methyl [1,2-$^{14}C_2$]-phenylacetate residue (1 g, 6.1 mmol) then was dissolved in anhydrous THF (10 mL). While stirring, this solution was slowly added to a solution prepared by adding n-butyllithium (0.7 mL, 7 mmol) to anhydrous THF (15 mL) containing diisopropylamine (7 mmol, 0.71 g) at -78°C and under an atmosphere of N_2 gas. After 1 hour of continuous stirring at -78°C, CH_3I (6 equivalents, 5.1 g) was added slowly under nitrogen gas and with continued maintenance of -78°C. The reaction mixture was gradually warmed to room temperature (over 1 h) and stirred overnight at room temperature under N_2 gas. Then THF was removed by evaporation under reduced pressure, after which the reaction residue was neutralized by the drop-wise addition of concentrated HCl. This solution was extracted with ethyl ether (5 x 20 mL) and the combined ether extracts were dried (anhydrous $MgSO_4$). Saponification of the methyl ester residue of (*RS*)-[1,2-$^{14}C_2$]-2-PPA was accomplished by dissolving the residue in methanol (30 mL) and by adding 3 mL of concentrated NaOH (1.5 g dissolved in 3 mL distilled water) followed by continuous refluxing (4 h). The methanol in the reaction mixture was evaporated under reduced pressure and the remaining aqueous phase acidified and then extracted with ethyl ether (5 x 20 mL). The ether extracts were combined, dried (anhydrous $MgSO_4$), and evaporated to dryness under reduced pressure. The clear oil residue was dissolved in 0.05 M phosphate buffer (8 mL) and the pH adjusted to 7.0 by the drop-wise addition of NaOH (1N). Purification of (*RS*)-[1,2-$^{14}C_2$]-2-PPA was achieved by reverse phase HPLC and

isocratic elution with a mobile phase consisting of 40% methanol in 0.05 M potassium phosphate buffer (pH 5.7) on a reverse-phase column (C_{18}, 150 x 4.6 mm, 5μ, 1 mL/min), detected by UV absorbance (226 nm). (*RS*)-[1,2-$^{14}C_2$]-2-PPA containing fractions were collected, acidified (1N HCl) and extracted with ethyl ether (4 x 40 mL). The combined ether extracts were dried (anhydrous $MgSO_4$) and evaporated to dryness leaving a clear colorless oil of (*RS*)-[1,2-$^{14}C_2$]-2-PPA (350 mg, 38.3 % yield). Reverse-phase HPLC (as above) followed by scintillation counting of HPLC fractions, collected every 30 seconds, showed the radiochemical purity of (*RS*)-[1,2-$^{14}C_2$]-2-PPA to be greater than 99%. ^{1}H NMR (deuterochloroform): δ 7.27-7.38 (m, 5H, phenyl ring), 3.74-3.80 (q, 1H, -CH-), 1.54-1.56 (d, 3H, $-CH_3$).

2.2.6 Synthesis of (*R*)-2-PPA-CoA, (*S*)-2-PPA-CoA, and (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA Thioesters

The synthesis and purification of acyl-CoA thioesters of (*R*)- and (*S*)-2-PPA were performed by conventional procedures employing ethyl chloroformate (25) exactly as we previously reported for the synthesis and purification of clofibryl-*S*-acyl-CoA thioester (Grillo and Benet, 2002). LC/MS analysis was performed by gradient elution from 5% to 100% acetonitrile over 20 min in 10 mM ammonium acetate (pH 5.0) on a Beckman C_8 reverse-phase column (150 x 4.6 mm, 5μ, 1 mL/min) and with a fragmentor voltage of 130. LC/MS analysis of the (*R*)- and (*S*)-2-PPA-CoA thioesters showed no presence of impurities, indicating only the presence of the acyl-CoAs eluting at 6.9 min for both anomers yielding identical mass spectra, *m/z* (%): *m/z* 900 (MH^+, 100%), *m/z* 393 ([M +

H – adenosinetriphosphate]$^+$, 84%), *m/z* 136 ([adenine + H]$^+$, 2%), *m/z* 428 ([adenosine diphosphate +2H]$^+$, 22%), *m/z* 291 ([M + H – 609]$^+$, 9%), *m/z* 491 ([M + H – 409]$^+$, 12%), and *m/z* 330 ([adenosine monophosphate]$^+$, 4%). Reverse-phase HPLC followed by scintillation counting of HPLC fractions, collected every 30 seconds, showed the radiochemical purity of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA to be 100 %.

2.2.7 Incubation Conditions

Incubations (1 ml total volume) of (*R*)- and (*S*)-2-PPA-CoA and 1-*O*-acyl glucuronide derivatives (at concentrations ranging from 15.6 to 500 nM) were performed in potassium phosphate buffer (0.05 M, pH 7.4) at 37°C in the presence or absence of GSH (10 mM). Incubations in the absence of GSH were performed to compare the chemical stability between the (*R*)- and (*S*)-2-PPA-CoA and 2-PPA-1-*O*-acyl glucuronide derivatives. For chemical stability experiments, aliquots (20 µL) of the incubation mixtures, performed in duplicate for each derivative, were taken at 0, 0.75, 1.5, 2.1 and 2.8 h for incubations containing acyl glucuronides and with continued outtakes at 5, 9 and 24 h for incubations containing acyl-CoA thioesters. During analogous incubations performed in the presence of GSH (10 mM) and 0.1 mM 2-PPA-CoA or 2-PPA-1-*O*-G derivatives, aliquots were taken at incubation times of 0, 0.5, 1, 2, 4 and 6 h, to determine time-dependent 2-PPA-SG formation. Incubations performed to determine concentration-dependent 2-PPA-SG formation were conducted in triplicate at 15.6, 31.3, 62.5, 125, and 500 nM 2-PPA-CoA or 2-PPA-1-*O*-G, with an incubation time of 5 min. Similar incubations were performed with (*R*)-2-PPA-CoA and (*R*)-2-PPA-1-*O*-G (500 nM) to determine, under identical conditions as above, the effect of varying GSH

concentration on 2-PPA-SG formation. Incubations were performed at 37°C and pH 7.4, in triplicate, with GSH concentrations of 10, 5, 1, 0.1, and 0.01 mM. For incubations of GSH with (*RS*)-2-PPA-*O*-acyl glucuronide migration isomers, a mixture 0.5 mL of 0.1 mM (*R*)- and 0.5 mL of 0.1 mM (*S*)-2-PPA-1-*O*-G in 0.05 M potassium phosphate (pH 7.4, 37°C) was allowed to degrade until there was no 1-*O*-acyl isomer left in the incubation (12 h), as determined by HPLC analysis with UV detection. Then GSH, followed by the careful drop-wise addition of NaOH (0.1N), was added to the incubation mixture to give a final GSH concentration of 10 mM and pH of 7.4. The rate of 2-PPA-SG formation was determined by LC-MS/MS and selected reaction monitoring (SRM) analysis. Aliquots of all incubations described above, were added directly to a quench solution (300 μL) consisting of 0.05 M potassium phosphate (pH 4.2) in methanol (1/1). Finally, 50 μL of this mixture was injected onto reverse-phase HPLC columns as described below.

2.2.8 HPLC Analysis

All reactions containing acyl-CoAs were analyzed on a C8 Zorbax column (150 x 4.6 mm, 5μ, 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. Analysis of 2-PPA-acyl glucuronides was performed by isocratic elution on a Microsorb-MV C18 column (150 x 4.6 mm, 5μ, Rainin LC and Supplies, 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. Analysis of 2-PPA-SG formed from the reaction of 2-PPA-CoA thioesters with GSH

was performed as described for the analysis of 2-PPA-CoA except that UV detection was performed at 226 nm. Analysis of 2-PPA-SG formed from the reaction of 2-PPA-1-*O*-acyl glucuronides with GSH was conducted by LC-MS/MS, with gradient elution (as describe above for the analysis of synthetic 2-PPA-SG), in positive ion mode, and by SRM of the MH^+ *m/z* 440 transition to the *m/z* 208 fragment (see Figure 2.6 later in this chapter).

2.2.9 Incubation of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA Thioester with Bovine Serum Albumin (BSA)

Fatty acid free BSA (50 mg/ml) was incubated with (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA (0.1 mM) in 0.05 M potassium phosphate buffer (10 mL, pH 7.4) at 37°C, and in triplicate. After 0, 0.5, 1, 2, 4, 6, 8 and 25 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 in 1.5 mL microcentrifuge tubes (10,000 *g*), after which the supernatant was discarded. The pellet was washed (15 x 1.5 mL) with a solution of 0.05 M potassium phosphate buffer and 7% perchloric acid (3:1, v/v) by vortex mixing and centrifugation as above until there was no radioactivity detected by scintillation counting of the resulting supernatants. The washing process continued by washing (10 x 1.5 mL) with a solution of ethanol and ethyl ether (3:1, v/v) until no radioactivity was detected in the supernatants after centrifugation. After the final supernatants were removed the washed pellets were left to dry at room temperature followed by the addition of NaOH (0.5 mL, 1 N) to the microcentrifuge tube containing the protein pellet. After heating at 80°C for 1 hour to dissolve the protein, 400 μL of the clear solution was analyzed by

scintillation counting. Protein concentrations were determined in 5 μL of the dissolved solution using BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as the standard, according to the manufacturer's instructions. Covalent binding is expressed as pmol 2-PPA bound per mg of BSA.

In experiments on the effect of pH on the covalent binding of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester to BSA after 25 h of incubation at 37°C, the pH of the solution was varied by adjusting the pH of a 0.05 M potassium phosphate solution with HCl (1N) or NaOH (1N). The pH values used were pH 5, 6, 7, 7.4, 8, 9 and 10. The chemical stability of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester was assessed at the 25 h time-point by taking 20 μL aliquots of the incubation mixture and adding them directly to a solution containing 0.05 M potassium phosphate (pH 4.2) and methanol (1:1, v/v). An injection volume of 50 μL of this solution was used for the measurement of remaining (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester and product (*RS*)-[1,2-$^{14}C_2$]-2-PPA by reverse-phase HPLC as described above.

In experiments performed to determine if the free sulfhydryl of BSA forms covalent adducts with 2-PPA, we incubated (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester (0.1 mM) in buffer (as above) with BSA (50 mg/mL) in the presence or absence of iodoacetic acid (1 mM, preincubation for 30 min at 37°C), a reagent that alkylates cysteinyl-thiol. Control incubations were performed where iodoacetic acid was replaced by sodium acetate (1 mM). Identical incubations were also performed with carboxymethylated-BSA (50 mg/mL). Incubations were performed (N = 3 for each treatment) at 37°C for 25 h, quenched, processed, and measured for covalently bound radioactivity as described above. Preliminary analysis was performed to determine the types linkages of (*RS*)-[1,2-

$^{14}C_2$]-2-PPA to BSA that occur. From incubations of BSA with (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA at 37°C, pH 7.4, and after 25 h of incubation, protein precipitates were washed exhaustively prior to further processing as follows: The protein pellets were either treated with hydroxylamine (1 M, pH 7.0, room temperature, 1.5 h) which specifically cleaves thioester and *O*-tyrosine esters, but not *O*-serine and *O*-threonine esters, or with KOH (0.1 M in 20% MeOH, room temperature, 1.5 h) which cleaves all esters (Sallustio et al., 2000). The treated protein precipitates then were acidified and processed as described above prior to analysis for covalently bound radioactivity and protein concentration.

2.3 Results

2.3.1 Chemical Stability Comparisons Between 2-PPA-CoA and 2-PPA-1-*O*-Acyl Glucuronides

Following the incubation of (*R*)- and (*S*)-2-PPA-CoA thioesters in potassium phosphate buffer (0.05 M) at pH 7.4 and 37°C, it was found that both of the CoA thioesters are chemically stable with no detectable hydrolysis occurring after 24 h of incubation (Figure 2.2). Experiments that were performed (data not shown) to determine the stability of (*R*)-2-PPA-CoA over 12 days of incubation and at varying pH showed that at pH 7.4 and 37°C the $t_{1/2}$ was 12 days, and at pH 10.5, the $t_{1/2}$ was 20.8 h. Experiments on the in vitro stability of 2-PPA-1-*O*-G in the absence of GSH showed that the acyl-glucuronides decomposed by 50% in 1.3 and 2.4 h of incubation at pH 7.4 and 37°C for (*R*)- and (*S*)-2-PPA-1-*O*-G, respectively (Figure 2.2).

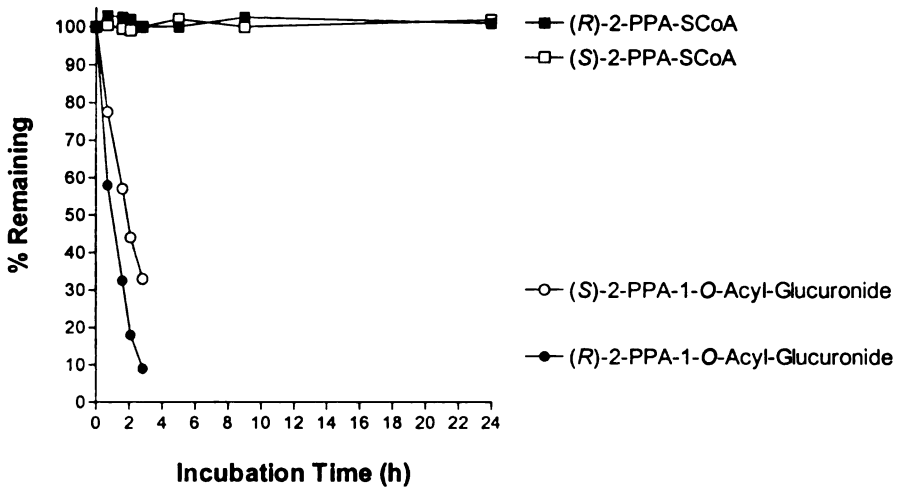


Figure 2.2 Time-course of the degradation of (*R*)- and (*S*)-2-PPA-CoA thioesters and acyl glucuronides (100 μM) in buffer (0.05 M potassium phosphate, pH 7.4, 37°C). Values are expressed as the average of duplicate incubations.

2.3.2 Reactions of GSH with 2-PPA-CoA and 2-PPA-1-O-Acyl Glucuronides

Incubation of (*R*)- or (*S*)-2-PPA-CoA (0.1 mM) with GSH (10 mM) led to the formation of 2-PPA-SG that eluted on HPLC with a retention time identical to the authentic 2-PPA-SG standard (5.8 min, Figure 2.3). Analysis by tandem mass spectrometry provided a mass spectrum of the product 2-PPA-SG identical to that acquired during the mass spectrometric analysis of authentic 2-PPA-SG (Figure 2.4). The tandem mass spectrum of the 2-PPA-SG reaction product (CID of MH^+ ion at *m/z* 440) showed characteristic fragment ions for glutathione conjugates (Baillie and Davis, 1993)

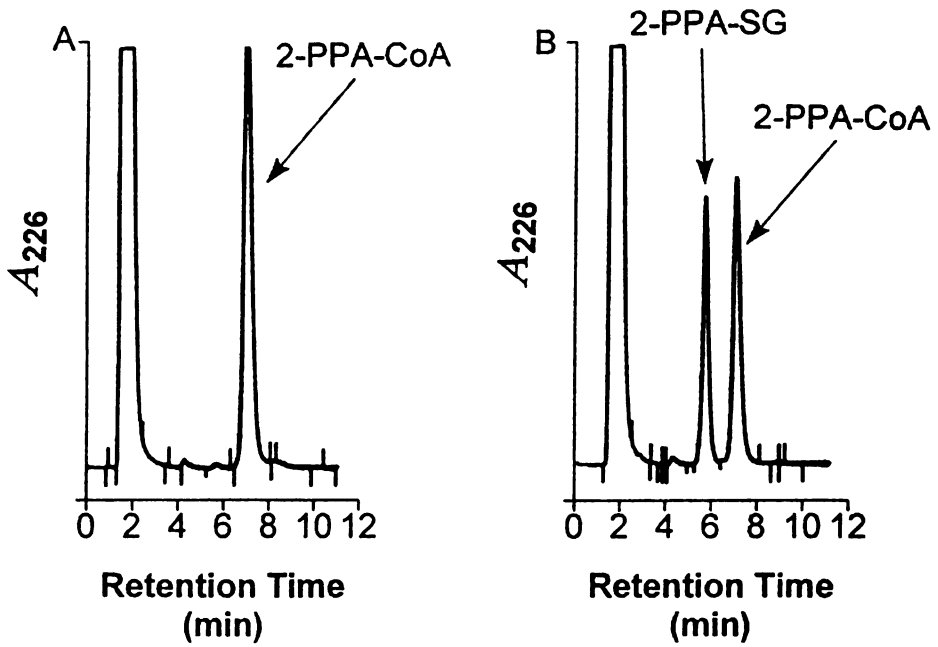


Figure 2.3 Representative reverse-phase HPLC chromatogram with UV detection of extracts from the reaction of (*R*)-2-PPA-CoA (100 μM, retention time 7.1 min) with GSH (10 mM) in buffer (0.05 M potassium phosphate, pH 7.4, 37°C). An aliquot of the incubation mixture (20 μL) was taken and added to 300 μL of MeOH:potassium phosphate buffer (0.05 M, pH 4.2), (1:1, v/v) after A) 0 and B) 4 h of incubation. Fifty μL of the quenched solution was injected onto a Beckman C8 column and analyzed by UV (A_{226}) detection. Shown in panel B is the 2-PPA-SG eluting at a retention time of 5.8 min.

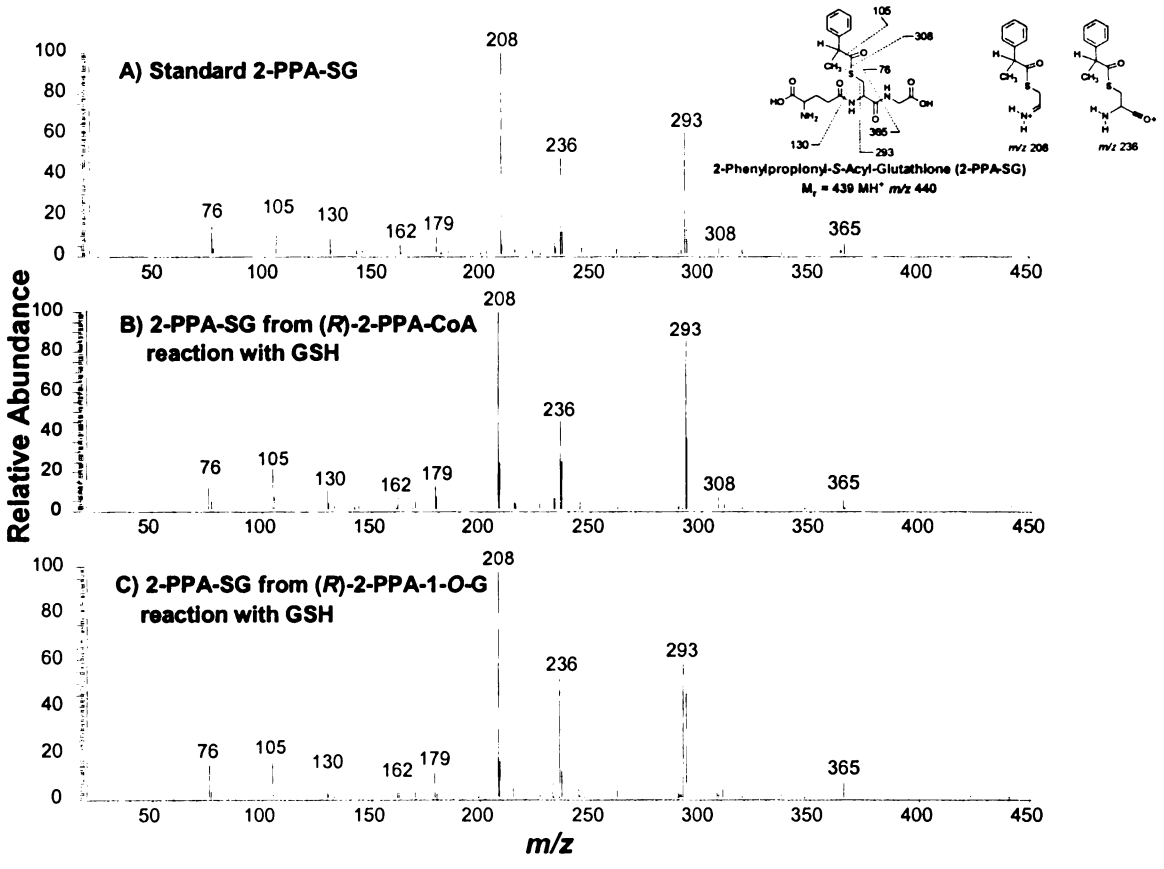


Figure 2.4 LC-MS/MS tandem mass spectra of A) Standard 2-PPA-SG, and 2-PPA-SG formed from the reaction of GSH with B) (*R*)-2-PPA-CoA and C) (*R*)-2-PPA-1-*O*-G.

including ions at *m/z* 365 ($[M + H - Gly]^+$, 8%), *m/z* 293 ($[M + H - pyroglutamic\ acid]^+$, 54%), *m/z* 236 ($[C_6H_5\text{-}CH(CH_3)COSCH_2CH{=}NH_2]^+$, 45%), *m/z* 208 ($[C_6H_5\text{-}CH(CH_3)COSCH_2CH(NH_2)(C{=}O)]^+$, 100%), *m/z* 130 ([pyroglutamic acid]+, 12%), *m/z* 105 ($[C_6H_5\text{-}CH(CH_3)]^+$, 14%), *m/z* 76 ($[glycine]^+$, 12%). The time-course for the reaction showed that the 2-PPA-SG was formed with an initial rate of 29.7 μM/h and reached a maximum of 77.6 μM after 6 h of incubation (Figure 2.5). The time-course of 2-PPA-SG formation during the reaction of (*R*)- or (*S*)-2-PPA-CoA with GSH was identical (data not

shown). Incubation of (*R*)- or (*S*)-2-PPA-1-*O*-G with GSH under the same conditions also resulted in the formation of 2-PPA-SG (Figures 2.4 and 2.6). Since the major fragment ion in the tandem mass spectrum was at *m/z* 208 (Figure 2.4), the transition of *m/z* 440 to *m/z* 208 was used in LC-MS/MS SRM for the analysis (Figure 2.6). The reaction occurred at an initial rate of 0.46 and 0.51 μM/h during reactions of GSH with (*R*)- and (*S*)-2-PPA-1-*O*-G, respectively (Figure 2.7). The acyl migration isomers of a racemic mixture of 2-PPA-1-*O*-G were also found to react with GSH (Figure 2.7) at an initial rate of 2-PPA-SG formation of 0.15 μM/h. In this reaction, a mixture (1:1, v/v) of 0.1 mM (*R*)- and 0.1 mM (*S*)-2-PPA-1-*O*-G in 0.05 M potassium phosphate (pH 7.4, 37°C) was allowed to degrade until there was no 1-*O*-acyl isomer left in the incubation (12 h), then GSH and NaOH (1N) were added to the incubation mixture to give a final GSH concentration of 10 mM and pH of 7.4.

Experiments to determine concentration-dependent 2-PPA-SG formation from the reaction of GSH (10 mM) with a range of 2-PPA acyl-CoA and 1-*O*-acyl glucuronide derivative concentrations (15.6 to 500 nM) showed a linear increase in 2-PPA-SG product formation with increasing concentration of the reactive acylating derivatives. Reactions were quenched after 5 min of incubation, where it was predicted that less that 5% of the starting 2-PPA-CoA or 2-PPA-1-*O*-G concentrations was depleted, which was based on the results from time-dependent GSH reaction experiments (Figures 2.5 and 2.7). Since the rate of 2-PPA-SG formation in the reactions of GSH of 2-PPA-CoA or 2-PPA-1-*O*-G changes in direct proportion to the change in concentration of either acylating species (2-PPA-CoA or 2-PPA-1-*O*-G, Figure 2.8) or GSH (Figure 2.9), then

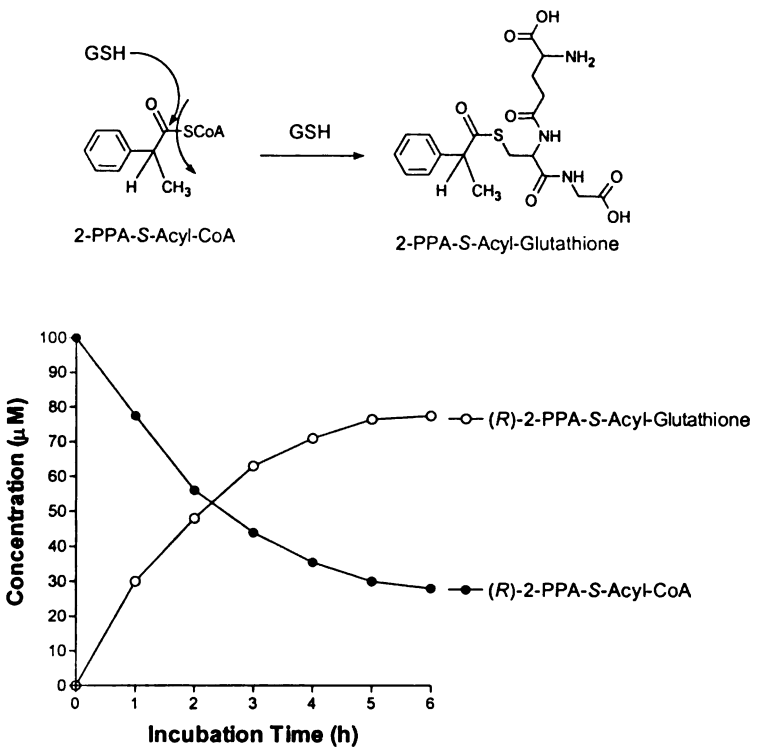


Figure 2.5 Time-course for the reaction of (*R*)-2-PPA-CoA (100 μM) with GSH (10 mM) forming 2-PPA-SG in buffer (0.05 M potassium phosphate, pH 7.4, 37°C). Values are expressed as the mean ± S.E. of triplicate incubations.

the rate equation for the reaction has the form: rate = *k*[acylating species] [GSH]. From the data shown in Figures 2.8 and 2.9, we calculated the second order rate constants to be $(1.9 \pm 0.2) \times 10^{-2}$ $M^{-1} \cdot s^{-1}$ from reactions of GSH with 2-PPA-CoA, and $(2.7 \pm 0.4) \times 10^{-4}$ $M^{-1} \cdot s^{-1}$ from reactions of GSH with 2-PPA-1-*O*-G. Therefore the reactivity of 2-PPA-CoA with GSH is 70.4 times greater than the reaction of 2-PPA-1-*O*-G with GSH. No enantioselective differences were found in the rate of reaction of either acylating species with GSH (Figure 2.8).

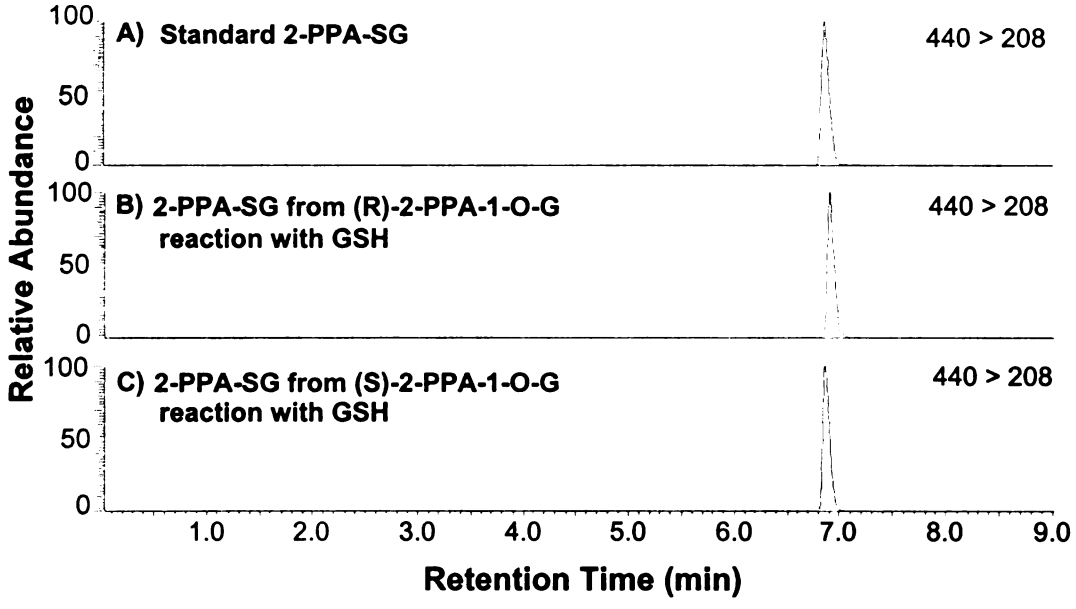


Figure 2.6 Representative reverse-phase gradient LC-MS/MS SRM chromatograms of A) standard 2-PPA-SG; and extracts of the reaction mixture of B) (*R*)-2-PPA-1-*O*-G and C) (*S*)-2-PPA-1-*O*-G with GSH (10 mM). The transition used for SRM analysis was *m/z* 440 > 208. As shown 2-PPA-SG eluted with a retention time of 6.95 min.

2.3.3 Transacylation of BSA by 2-PPA-CoA In Vitro

Incubations of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester (0.1 mM) with fatty acid free BSA (50 mg/mL) were carried out in potassium phosphate buffer (0.05 M) at indicated

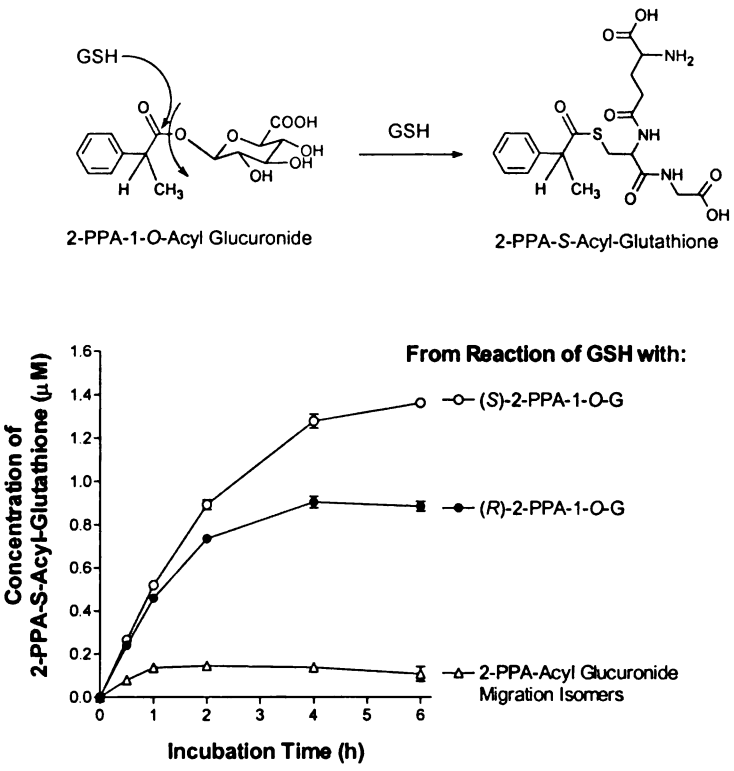


Figure 2.7 Time-course for the reaction of (*R*)-2-PPA-1-*O*-G, (*S*)-2-PPA-1-*O*-G, and racemic 2-PPA-acyl glucuronide migration isomers (100 μM) with GSH (10 mM) forming 2-PPA-SG in buffer (0.05 M potassium phosphate, pH 7.4, 37°C). Values are expressed as the mean ± S.E. of triplicate incubations.

pH values and at 37°C. Measurements of the stability of 2-PPA-CoA in the same incubations were determined by HPLC analysis. Covalent binding to BSA was measured by analysis of radioactivity irreversibly bound to exhaustively washed protein precipitates. The extent of covalent binding of 2-PPA, via 2-PPA-CoA, to BSA was

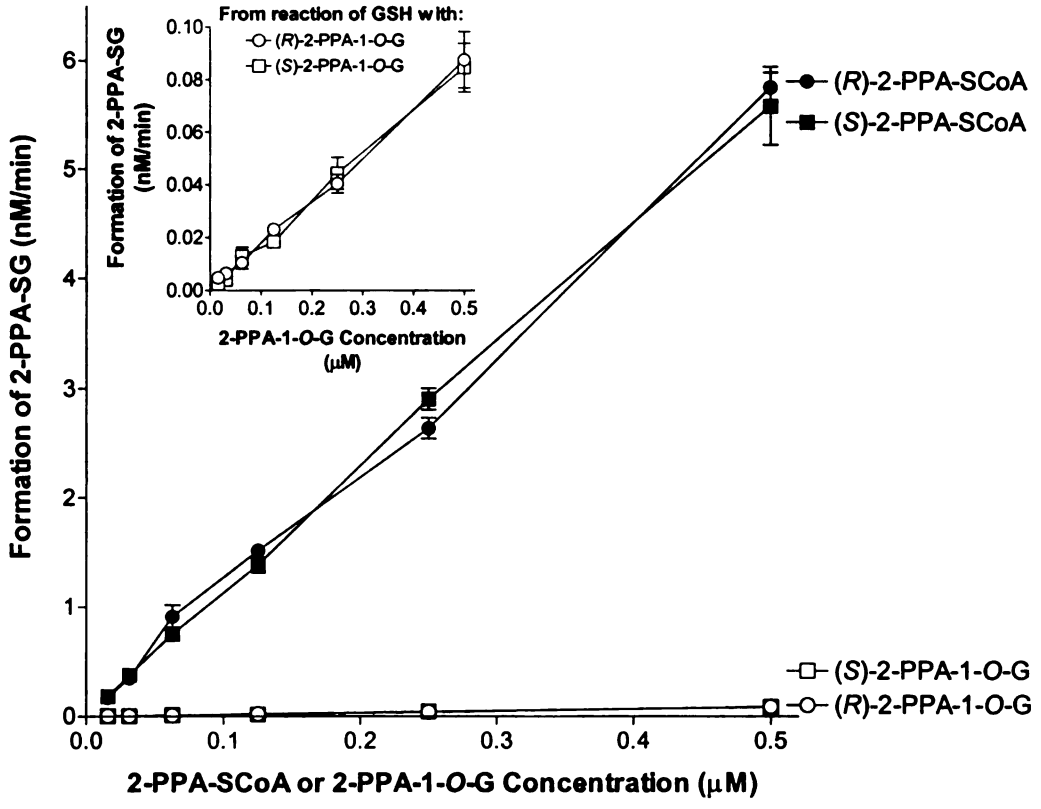


Figure 2.8 Effect of varied 2-PPA-CoA or 2-PPA-1-*O*-G concentrations on the rate of 2-PPA-SG formation during reactions with GSH (10 mM) at pH 7.4 and 37°C. The inset shows part of the graph where the y-axis has been expanded to show the rate of reaction of 2-PPA-1-*O*-G with GSH. Values are expressed as the mean ± S.E. of triplicate incubations.

time- and pH-dependent and the rate of covalent adduct formation (3 pmol/mg BSA·h) was roughly linear over 25 h of incubation (Figure 2.10). The covalent binding to BSA increased with increasing pH, from 19 pmol/ mg BSA at pH 5.0 to 376 pmol/mg BSA at pH 10 after incubation for 25 h at 37°C (Figure 2.11). This pH-dependent covalent binding correlated well with the enhanced hydrolysis of 2-PPA-CoA to the free acid with

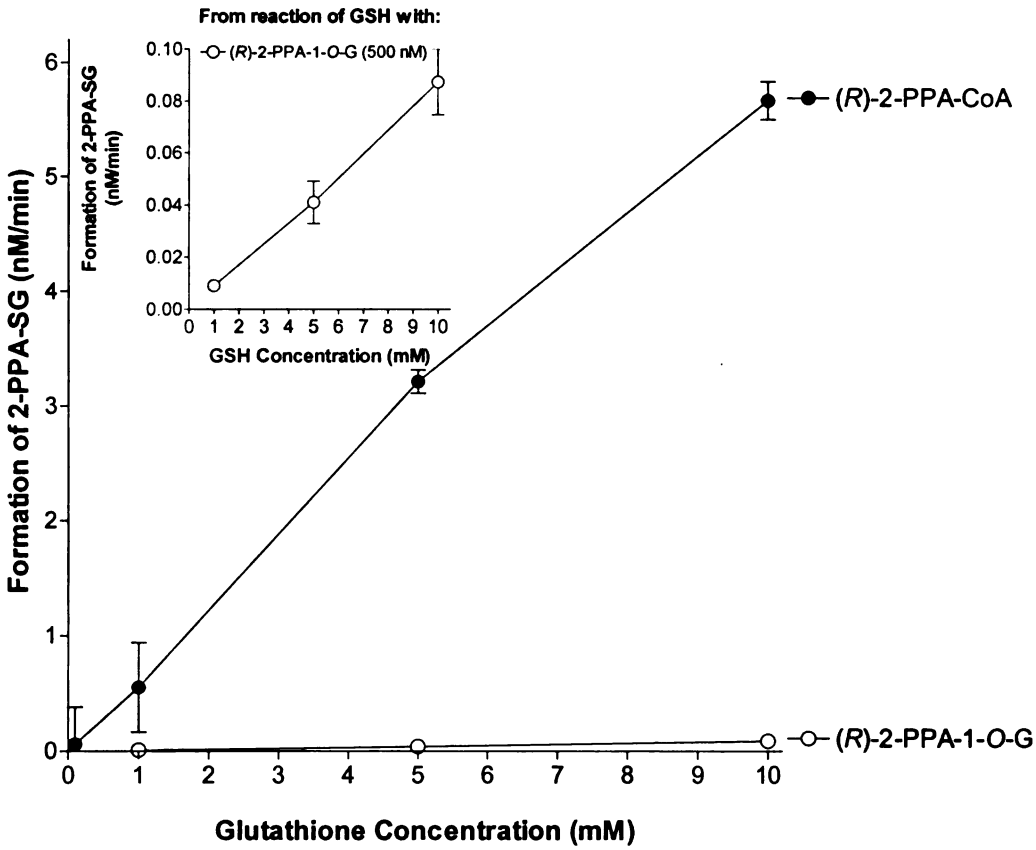


Figure 2.9 Effect of varied GSH concentration on the rate of 2-PPA-SG formation during reactions with (*R*)-2-PPA-CoA or (*R*)-2-PPA-1-*O*-G (500 nM) at pH 7.4 and 37°C. The inset shows part of the graph where the y-axis has been expanded to show the rate of reaction of (*R*)-2-PPA-1-*O*-G with GSH. Values are expressed as the mean ± S.E. of triplicate incubations.

increasing incubation pH. The free cysteinyl-sulfhydryl of BSA was not critically important for the majority of covalent binding of 2-PPA to BSA, since covalent binding only decreased by ~18 % in incubations where BSA was treated with iodoacetic acid (a reagent that alkylates reduced thiols), and because incubations with *S*-carboxymethyl-BSA resulted in covalent binding levels that were not significantly different than control

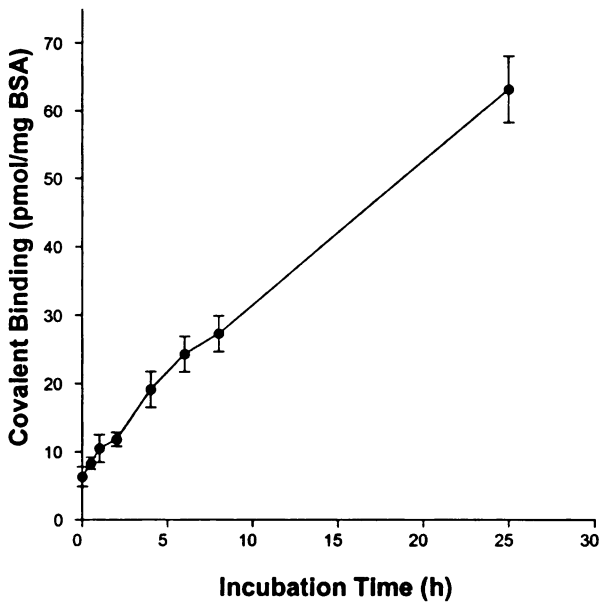


Figure 2.10 Time-dependent covalent binding of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA (0.1 mM) to BSA (50 mg/mL) in potassium phosphate buffer (0.05 M, pH 7.4) at 37°C. Values represent means ± S.E. from triplicate incubations.

(Table 2.1). Experiments performed to determine the types of 2-PPA-BSA adduct linkages utilized hydroxylamine (1 M at pH 7.0), which selectively hydrolyzes thioester and *O*-tyrosine ester linked adducts, and KOH (0.1 M) with 20% methanol, which hydrolyzes all ester linkages including *O*-tyrosine and *O*-serine/-threonine esters (Sallustio et al., 2000). Hydroxylamine or KOH/methanol treatments of protein precipitates containing covalently bound 2-PPA yielded 44.1 ± 1.7 % and 40.6 ± 6.6% loss, respectively, of bound radiolabel. Together these results suggest that 2-PPA-CoA

acylates BSA at pH 7.4 and 37°C forming primarily *O*-tyrosine- and amide-linked adducts.

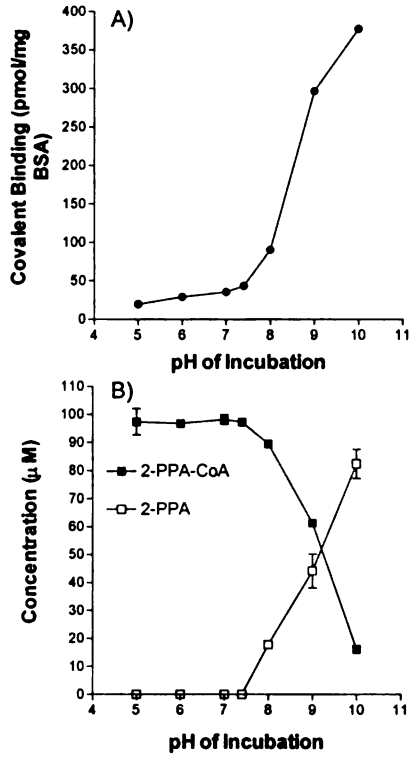


Figure 2.11 The effect of pH on the A) covalent binding to BSA and B) chemical stability of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester (0.1 mM) in 0.05 M potassium phosphate buffer containing BSA (50 mg/mL) after 25 h of incubation. Values represent means ± S.E. from triplicate incubations.

Table 2.1 Identification of thioester, amide, or oxygen ester linked protein adducts. The effect of *S*-alkylated BSA on the covalent binding of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA to BSA, and the effect of post-incubation treatment of protein adducts by selective hydrolysis.

Incubations:	**Covalent Binding of (RS)[1,2-$^{14}C_2$]-2-PPA-CoA to BSA (37°C, pH 7.4, 25 h)** *% Control*
BSA + Iodoacetic acid (1 mM)	81.7 ± 4.9*
BSA + Acetic acid (1 mM)	93.1 ± 14.1
S-Carboxymethyl-BSA	116.7 ± 6.6
Post-Incubation Treatment of Control Protein Pellets:	
Hydroxylamine (1 M, pH 7.0) (Cleaves thioester and *O*-tyrosine esters.)	55.9 ± 1.7*
KOH (0.1 M, 20% MeOH) (Cleaves all esters.)	59.4 ± 6.6*

Values represent means ± S.E. from triplicate incubations. *p <0.05, different from control.

2.4 Discussion

Carboxylic acid-containing drugs that have been shown to cause idiosyncratic allergic reactions in humans have been proposed to do so by way of immune-based reactions to covalently bound drug protein conjugates (Faed, 1984; Spahn-Langguth and Benet, 1992; Boelsterli et al., 1995). Reactive acyl glucuronide metabolites of many acidic drugs are known to react with protein in vitro by two alternative routes. One of these routes is the transacylation of protein nucleophiles, such as sulfhydryl, hydroxyl and amino groups, leading to thioesters, oxygen esters and amide-linked protein adducts, respectively (Qiu et al., 1998b). Another mechanism involves the acyl-migration of 1-*O*-acyl-glucuronides to their 2-, 3-, and 4-*O*-acyl isomers, followed by a ring-chain tautomerism providing the straight chain aldehydes, which finally can form Schiff-base

type adducts with protein lysines (Ding et al., 1995; Qiu et al., 1998b). These adducts can be made irreversible by undergoing an Amadori rearrangement reaction to stable 1-amino-2-keto products (Ding et al., 1995). In addition to a range of reactive acyl glucuronides that have been examined, the chemical reactivity of a number of *S*-acyl-CoA thioester metabolites of acidic drugs are increasingly being characterized. Such studies include the recent report on the reactivity of nafenopin-CoA with human liver microsomal protein in vitro (Sallustio et al., 2000), the acylation of GSH by clofibryl-*S*-acyl-CoA in vitro (Grillo and Benet, 2002), and the non-enzymatic autoacylation of protein by palmitoyl-CoA (Bradberry et al., 2000).

The present studies were focused on determining the relative ability of acyl glucuronide and acyl-CoA thioester metabolites of 2-PPA to transacylate the nucleophilic cysteinyl-thiol of GSH in vitro in buffer. We propose that the greater the reactivity of the acylating metabolite with GSH in vitro, the higher the probability that the metabolite may acylate protein nucleophiles in vivo, and thus the higher the potential toxicity.

Here, we first examined the relative stabilities of 2-PPA acyl glucuronides and acyl-CoA derivatives in vitro in buffer under physiological conditions (pH 7.4 and 37°C). Results showed that both (*R*)- and (*S*)-2-PPA-CoA derivatives are very stable, showing no detectable hydrolysis (Figure 2.2), yielding an apparent $t_{1/2}$ of 12 days. These results are similar to the reported high chemical stability of clofibryl-*S*-acyl-CoA in buffer, where only minimal hydrolysis occurs under very similar conditions (Grillo and Benet, 2002). By contrast, the 1-*O*-acyl glucuronide derivatives of 2-PPA were unstable, as indicated by the relatively short $t_{1/2}$'s of 1.3 h and 2.4 h for (*R*)-2-PPA-1-*O*-G and (*S*)-2-PPA-1-*O*-G, respectively. These data are in close agreement with a recent report

showing that the overall degradation $t_{1/2}$ of (*R*)- and (*S*)-PPA-1-*O*-acyl glucuronides was 1.8 and 3.3 h, respectively (Akira et al., 2000). In addition, the authors of that study determined that acyl migration predominated over hydrolysis. Together, these data indicate that the $t_{1/2}$ of (*R*)-2-PPA-1-*O*-acyl glucuronide is approximately 54% of the $t_{1/2}$ for the (*S*)-antipode. The increased instability of the (*R*)-2-PPA-1-*O*-acyl isomer compared to the (*S*)-antipode is consistent with our chemical stability reports on a range of profen 1-*O*-acyl glucuronides (Li and Benet, 2002). Studies on the chemcial reactivity of clofibryl-1-*O*-acyl glucuronide have shown that once acyl migration occurs, forming the 2-, 3-, and 4-*O*-acyl isomers, the glucuronide no longer reacts with glutathione (Shore et al., 1995). Therefore, as with clofibryl acyl glucuronide, acyl migration of 2-PPA-1-*O*-G leads to isomers less able to acylate GSH.

The present studies on the reactivity of 2-PPA-CoA (0.1 mM) with GSH (10 mM) in buffer at pH 7.4 and 37°C showed the formation of 2-PPA-SG thioester (Figure 2.3) to be time-dependent, resulting in approximately 77 μM 2-PPA-SG formed after 6 h of incubation (Figure 2.5). Formation rates of 2-PPA-SG from reactions of GSH with (*R*)- or (*S*)-2-PPA-CoA thioesters were identical, indicating no stereoselectivity in the transacylation-type reaction. Tandem mass spectrometric analysis by of the 2-PPA-SG product (CID of MH^+ at *m/z* 440) provided a mass spectrum consistent with the structure of the 2-PPA-*S*-acyl glutathione derivative. Characteristic fragment ions for glutathione adducts (Baillie and Davis, 1993) were observed, such as the loss of pyroglutamic acid (-129 amu), and the product ions at *m/z* 208, 236 and 293, which indicated that the 2-PPA portion of the conjugate was covalently bound to the cysteinyl-thiol, rather than the γ-glutamyl-amine of glutathione.

In vitro reactions of (*R*)-and (*S*)-2-PPA-1-*O*-G with GSH, under identical conditions as the reactions of GSH with 2-PPA-CoA, also resulted in time-dependent formation of 2-PPA-SG (Figures 2.4, 2.6, and 2.7). The initial rate of the reaction with GSH forming 2-PPA-SG was not stereoselective (nearly 0.25 μM formed in both reactions after 30 min of incubation), but after 1 h of incubation, the reaction of GSH with the (*S*)-antipode resulted in more transacylation of GSH forming 2-PPA-SG. It was also determined that the acyl migration isomers of (*RS*)-2-PPA-1-*O*-G were able to react with GSH, but at an initial rate that was approximately 30% of that determined for the 1-*O*-acyl isomers. This is the first report demonstrating the ability of acyl migration isomers of an acyl glucuronide to transacylate GSH, and is in contrast to studies showing that the acyl migration isomers of clofibryl glucuronide, under similar conditions, did not transacylate GSH forming clofibryl-*S*-acyl-glutathione (Shore et al., 1995). The decreased overall 2-PPA-SG formed upon incubation of GSH with the (*R*)-1-*O*-acyl glucuronide compared to the (*S*)-isomer, probably reflects the greater acyl migration rate of the (*R*)-isomer to less reactive acyl migration isomers. Overall, the results from these experiments show a more rapid and extensive reaction of the *S*-acyl-CoA thioesters of 2-PPA with GSH than the reaction with the respective 1-*O*-acyl glucuronides. Concentration-dependent experiments, where the concentrations of 2-PPA acylating species (Figure 2.8) or GSH (Figure 2.9) were varied, showed that reactions of GSH with 2-PPA-CoA and 2-PPA-1-*O*-G are second order reactions in the formation of 2-PPA-SG. Also the results showed that 2-PPA-CoA transacylates GSH with 70.4 times greater reactivity than does 2-PPA-1-*O*-G. These data are consistent with recent studies showing that clofibryl-*S*-acyl-CoA was nearly 40-fold more reactive with GSH, forming clofibryl-

S-acyl-glutathione (Grillo and Benet, 2002), than was the same reaction with clofibryl-1-*O*-acyl glucuronide (Shore et al., 1995).

In another set of experiments, we reacted (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA thioester with BSA, used here as a model protein, in vitro to determine the ability of 2-PPA-CoA to transacylate the protein. Results showed that 2-PPA became covalently bound to BSA in a linear and time-dependent fashion at a rate of 3 pmol 2-PPA/mg BSA.h and reached approximately 63 pmol 2-PPA/mg BSA after 25 h of incubation (Figure 2.10). The covalent binding determined at the 0 h time-point (~6 pmol 2-PPA/mg BSA) may reflect an initial rapid acylation of protein, or the inability to wash away completely all of the non-covalently bound 2-PPA-CoA. The covalent binding of 2-PPA to protein in these incubations was determined to be dependent also on the pH of the incubation mixture (Figure 2.11). Covalent binding, even though 2-PPA-CoA was being degraded more rapidly, was greater after 25 h of incubations with increasing pH, indicating the higher reactivity with protein tyrosine, sulfhydryl and amine residues that become more nucleophilic with increasing pH.

The effect of blocking of the free sulfhydryl of BSA, Cys-34, with iodoacetic acid (Peters, 1985), led to a significant but small decrease in covalent binding of 2-PPA to BSA (18.3% decrease), but no significant difference in the covalent binding was observed when BSA was treated with 1 mM acetic acid. Since the pH of the incubation is important for covalent binding (Figure 2.11), care was taken to make sure that the pH of the incubations treated with iodoacetic acid or acetic acid was pH 7.4 before the addition of (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA to the reaction mixture. The lack of importance for the free Cys-34 sulfhydryl in the covalent binding to (*RS*)-[1,2-$^{14}C_2$]-2-PPA-CoA was

shown when there was no decrease in covalent binding to protein in incubations with *S*-carboxymethyl-BSA. The effect of hydroxylamine hydrolysis (which cleaves thioesters and *O*-tyrosine esters at neutral pH), as well as the effect of alkaline methanolysis (which cleaves all esters) on the extent of covalent binding of 2-PPA to BSA showed that both treatments led to very similar decreases (41-44%) in covalently bound 2-PPA. Based on these results, and on the results with *S*-carboxymethyl-BSA, 2-PPA-BSA adducts appear to be primarily linked via *O*-tyrosine ester (44%) and amide bonds (41%). Similar types of adducts were found in incubations of nafenopin with human liver microsomes (Sallustio et al., 2000) in the presence of cofactors for acyl-CoA formation (ATP, CoA, Mg^{2+}.

In summary, the results from these studies show that both the 1-*O*-acyl glucuronide and 2-PPA-*S*-acyl-CoA metabolites of 2-PPA are chemically reactive species that can transacylate the cysteinyl-thiol of glutathione. The superior ability of the 2-PPA-CoA thioester derivative to transacylate GSH, versus the respective 1-*O*-acyl glucuronide, may indicate that *S*-acyl-CoA metabolites of 2-arylpropionic acid drugs, in general, are as or more important than their respective acyl glucuronides in the covalent binding of these drugs to protein in vivo. Since 2-PPA-CoA was shown to react covalently with BSA in these experiments, we propose that acidic drugs that are metabolized to acyl-CoA thioesters can covalently bind to protein in vivo through this mechanism.

Chapter 3

Covalent Binding of 2-Phenylpropionyl-*S*-Acyl CoA Thioester to Tissue Proteins In Vitro

3.1 Introduction

2-Arylpropionic acids (profens) are a commonly used class of non-steroidal anti-inflammatory drugs (NSAIDs), widely prescribed as analgesic, antipyretic and anti-inflammatory agents. Although profen drugs are generally well tolerated, in rare cases the use of profen drugs has been associated with the development of severe and sometimes fatal hypersensitivity reactions, such as allergic reactions and drug-induced organ toxicity (Zimmerman, 1994; van der Klauw et al., 1996). Several profen drugs including benoxaprofen, indoprofen, pirprofen and suprofen have been withdrawn from the market due to severe idiosyncratic toxicities (Bakke et al., 1984; Bakke et al., 1995). The mechanism responsible for the initiation of such toxic side effects remains poorly understood. Covalent modification of tissue proteins by reactive metabolites of profen drugs is often suggested as a possible mechanism to mediate some of these idiosyncratic toxicities (Boelsterli et al., 1995; Pumford et al., 1997).

Acyl glucuronides are generally believed to be important reactive metabolites of profen drugs and potentially responsible for covalent adduct formation in vivo (Figure 3.1). 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 pharmacological active parent drug and that also undergo intramolecular acyl migration to yield β-glucuronidase resistant isomers (Spahn-Langguth and Benet, 1992; Li and Benet, 2002). More importantly, these electrophilic metabolites can bind covalently to serum albumins in vitro and to plasma and tissue proteins in vivo (Smith et al., 1986; Li and Benet, 2002).

An alternative pathway that may lead to formation of reactive metabolites of profen drugs is the acyl-Coenzyme A (acyl-CoA) pathway (Figure 3.1). Enantioselective formation of acyl-CoA derivatives is believed to be the key step for the uni-directional chiral inversion of profen drugs from the pharmacologically inactive (*R*)- to the active (*S*)-enantiomer (Caldwell et al., 1988; Hall and Quan, 1994). The activated acyl-CoA derivatives of profen drugs could also participate in lipid metabolism pathways resulting in the formation of hybrid triacylglycerols and cholesteryl esters (Figure 3.2) (Fears, 1985). Acyl-CoA thioesters are not excreted, but are further metabolized to glycine, carnitine and taurine conjugates, processes requiring a reactive acyl-CoA thioester bond (Hutt and Caldwell, 1990).

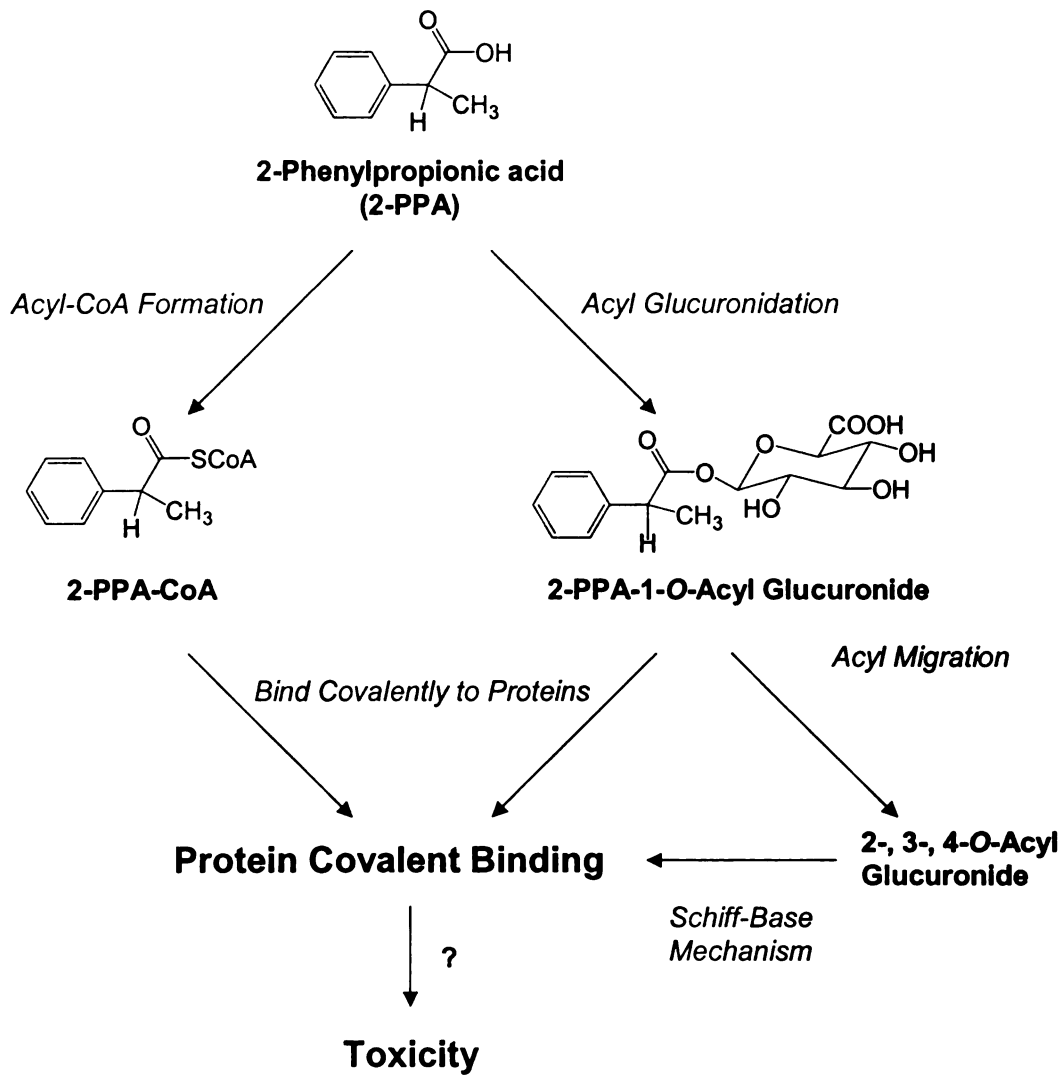


Figure 3.1 Two alternative metabolic activation pathways of 2-phenylpropionic acid, namely acyl-CoA formation and acyl glucuronidation, and their potential involvement in covalent binding to proteins.

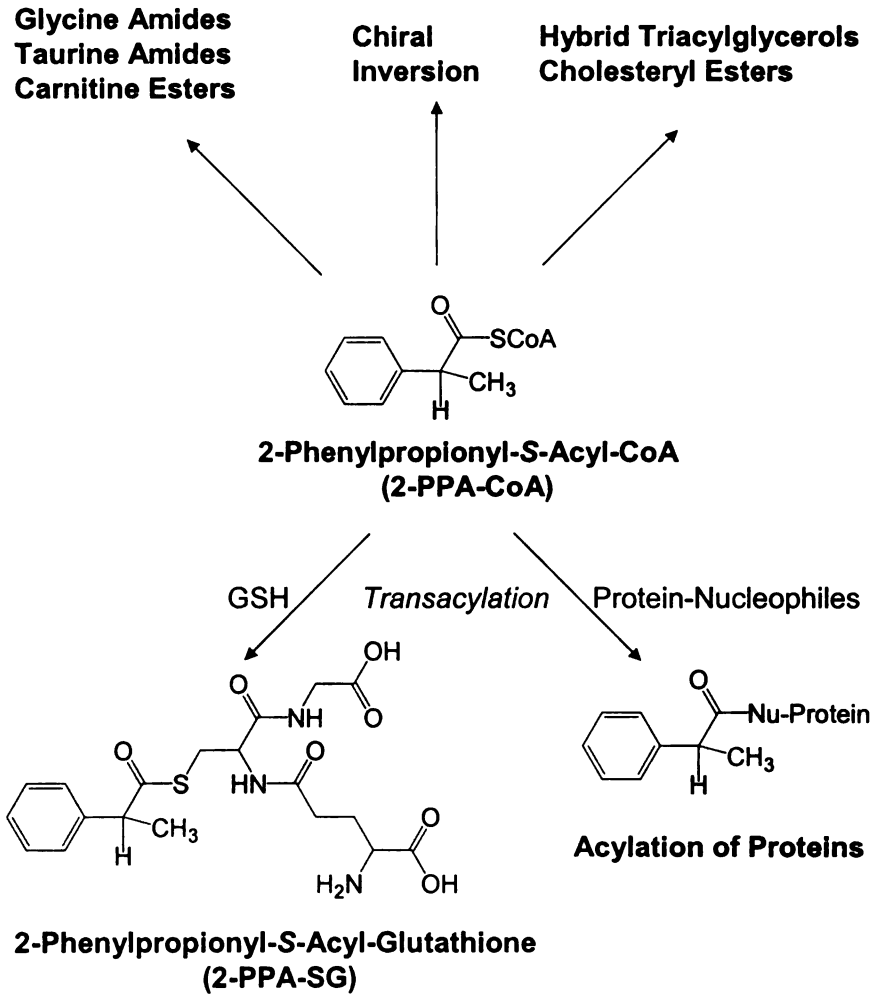


Figure 3.2 Schematic representation of the potential metabolic fate of 2-PPA-CoA thioester.

Like acyl glucuronides, thioester-linked acyl-CoA derivatives are 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 acid acylation have shown that

endogenous acyl-CoAs, including palmitoyl-CoA and arachidonoyl-CoA, can react non-enzymatically with sulfhydryl groups on proteins and peptides in vitro in a time- and concentration-dependent fashion (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). Since acyl-CoA thioesters of profen drugs possess the same reactive thioester-linked carbonyl-carbon, it is possible that acyl-CoA derivatives of profen drugs might also acylate proteins. However, studies on the reactivity of acyl-CoA thioesters of profen drugs have rarely been performed. Our recent studies with 2-phenylpropionic acid (2-PPA, the simplest congener of profen drugs) demonstrated that 2-phenylpropionyl-*S*-acyl-CoA (2-PPA-CoA) was able to acylate glutathione (GSH) sulfhydryl to form 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) (Chapter 2 and Li et al., 2002). 2-PPA-1-*O*-G has been shown to be reactive and able to bind covalently to serum albumin in vitro (Georges et al., 1999; Akira et al., 2000). However, to date, there is no information on the chemical reactivity of 2-PPA-CoA towards proteins. The present study was designed to characterize such chemical reactivity of 2-PPA-CoA thioester and examine its possible involvement in protein adduct formation in rat liver homogenate and to human serum albumin (HSA) in buffer. Results from these in vitro studies strongly suggest that acyl-CoA thioesters of profen drugs are chemically reactive electrophiles able to bind covalently to tissue proteins in vitro. They may, therefore, provide an additional mode for covalent binding of profen drugs in vivo.

3.2 Experimental Section

3.2.1 Chemicals

2-PPA, (*R*)-(-)-2PPA, (*S*)-(+)-2-PPA, ethyl chloroformate, perchloric acid (70%) and ethylenediaminetetraacetate (EDTA) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). CoA, ATP, $MgCl_2$, 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 Co. (St. Louis, MO). Potassium phosphate monobasic and THF (anhydrous) were purchased from Fisher Scientific (Fair Lawn, NJ). Hionic-Fluor scintillation fluid was purchased from Packard BioScience Co. (Meriden, CT). (*RS*)-[1-^{14}C]-2-PPA (50 mCi/mmol) was synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO). (*RS*)-[1-^{14}C]-2-PPA-CoA and authentic (*R*)-2-PPA-CoA were synthesized as described below. All solvents used for HPLC analysis were of chromatographic grade.

3.2.2 Synthesis of (*R*)-2-PPA-CoA Thioester and (*RS*)-[1-^{14}C]-2-PPA-CoA

Synthesis and purification of the acyl-CoA thioester of 2-PPA were performed by conventional procedures employing ethyl chloroformate, as reported previously for the synthesis and purification of clofibryl-*S*-acyl-CoA thioester (Grillo and Benet, 2002). LC/MS analysis was performed by gradient elution from 5% to 100% acetonitrile over 20 min in 10 mM ammonium acetate (pH 5.0) on a Beckman C_8 reverse-phase column (150 x 4.6 mm, 5μm, 1 mL/min) using a fragmentor voltage of 130. LC/MS analysis of the

(*R*)-2-PPA-CoA thioester showed no impurities and only the acyl-CoA eluting at 6.9 min: *m/z* 900 (MH^+, 100%), *m/z* 393 ([M + H – adenosinetriphosphate]$^+$, 84%), *m/z* 428 ([adenosine diphosphate +2H]$^+$, 22%), *m/z* 491 ([M + H – 409]$^+$, 12%), *m/z* 291 ([M + H – 609]$^+$, 9%), *m/z* 330 ([adenosine monophosphate]$^+$, 4%) and *m/z* 136 ([adenine + H]$^+$, 2%). Reverse-phase HPLC followed by scintillation counting of HPLC fractions, collected every 30 seconds, showed the radiochemical purity of (*RS*)-[1-^{14}C]-2-PPA-CoA to be 100 %.

3.2.3 Incubation of (*RS*)-[1-^{14}C]-2-PPA-CoA with Human Serum Albumin (HSA)

Fatty acid free HSA (30 mg/mL) was incubated with (*RS*)-[1-^{14}C]-2-PPA-CoA (0.1 Ci/mol, 0.1 mM) or (*RS*)-[1-^{14}C]-2-PPA (0.1 Ci/mol, 0.1 mM, negative control) in 0.05 M potassium phosphate buffer (10 mL, pH 7.4) at 37°C, and 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 (1500 *g*) in 15 mL Falcon polypropylene conical tubes for 10 min. The supernatants were neutralized by 1 M NaOH and analyzed by reverse-phase HPLC for the stability of 2-PPA-CoA thioester. The pellet was washed (10 x 5 mL) with a solution of 0.05 M potassium phosphate buffer (pH 4.5) and 7% perchloric acid (3:1, v/v) by vortex mixing and centrifugation as above until no radioactivity was detected by scintillation counting of the resulting supernatants. The washing process was continued with a mixture of methanol and ethyl ether (3:1, v/v, 10 x 5 mL) until no radioactivity was detected in the supernatants after centrifugation. After the final supernatants were removed, the washed pellets were left to dry at room temperature followed by the

addition of 1 M NaOH (0.5 mL) to the conical tube containing the protein pellet. The mixture was heated at 80°C overnight to hydrolyze the protein. The radioactivity of 0.4 mL of the clear solution was counted in 10 mL scintillation fluid. Protein concentrations were estimated using the BCA protein assay reagent kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard, according to the manufacturer's instructions. Covalent binding is expressed as pmol 2-PPA bound per mg of HSA.

3.2.4 Animals and Tissue Collection

Male Sprague-Dawley rats (240~260 g) were purchased from B& K Universal (Livermore, CA) and maintained in a controlled housing environment with 12-h light/dark cycles and fed standard laboratory chow for ≥3 days before they were killed. All animal studies were approved by the University of California San Francisco Committee on Animal Research. Rats 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. Frozen livers were stored at -80°C until preparation of tissue homogenate.

3.2.5 Preparation of Rat Liver Homogenate

Pooled tissue homogenates were prepared from livers of six rats as described previously (Tracy et al., 1993) with minor modifications. Briefly, frozen rat livers were thawed, cut into pieces and quickly weighed. Three times tissue weight (in volume) of homogenate buffer (50 mM Tris-HCl, 0.15 M KCl, 1 mM EDTA, and 1 mM DTT

(fresh), pH 7.4) was then added and the livers homogenized at 0°C with a Brinkmann homogenizer (model PT 10/35, Brinkmann, Westbury, NY) set at speed level 5 for two 20 s intervals. The homogenate centrifugated for 15 min at 2000 *g* and the supernatant, referred to as the whole homogenate fraction, was then transferred to plastic tubes and stored at -80°C. Protein concentrations were measured by the Pierce BCA protein assay (Pierce Chemical, Rockford, IL) with bovine serum albumin (BSA) as the standard.

3.2.6 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 $MgCl_2$ and 2.5 mM ATP in a final volume of 0.5 mL. After a 3 min preincubation, reactions were initiated by addition of ATP and allowed to proceed for 30 min at 37°C, then stopped by adding 50 µL perchloric acid (7%). After centrifugation at 10,000 *g* for 5 min, the supernatants were neutralized with 1 M NaOH and analyzed by reverse-phase HPLC. Formation of 2-PPA-CoA was linear with incubation time and protein concentration and the enzymes were saturated with the cofactors (CoA and ATP) under the above incubation conditions. Values for K_m and V_{max} were determined by an extended least-square regression modeling program (WinNonlin Software).

3.2.7 Covalent Binding Studies with Rat Liver Homogenate

Incubations were carried out in triplicate at 37°C in 150 mM Tris-HCl (pH 7.4) containing 1.0 mM (*RS*)-[1-^{14}C]-2-PPA (2 Ci/mol), 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM $MgCl_2$, 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 10,000 *g* for 5 min, the supernatants were neutralized with 1 M 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 previously (Sallustio et al., 2000) with minor modifications. Briefly, the protein pellets were washed with 2 mL of a solution consisting of two parts of 4% (v/v) acetic acid in acetonitrile (ACN) and 1 part of 10 mM potassium phosphate buffer (pH 4.5), mixed vigorously for 5 min and centrifuged (10,000 *g*, 5 min). The wash procedures were repeated (15 times) until there was no detectable radioactivity in the supernatant. The remaining protein pellets were dissolved in 1 M NaOH (0.2 mL) at 80°C overnight. The clear solution was counted in 10 mL of Hionic-Fluor scintillation fluid. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as the standard, according to the manufacturer's instructions. Covalent binding is expressed as pmol bound 2-PPA per mg of protein.

3.2.8 Inhibition of ^{14}C-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. The inhibitory effects of these four acids on the 2-PPA-CoA formation and covalent binding were studied in rat liver homogenate. 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 to incubation tubes and then dried under nitrogen before addition of the remaining incubation components. All incubation series included negative controls lacking ATP.

3.2.9 HPLC Analysis

HPLC analysis was carried out on a Shimadazu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadazu Corp., Japan) with a Shimadazu SCL-10A controller and a Shimadazu SPD-10A UV-Vis detector. The formation of 2-PPA-CoA in rat liver homogenates was analyzed on a SB-C_8 Zorbax column (150 x 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% ACN in 0.19 M ammonium acetate buffer (pH 7.0) was used with UV detection at 262 nm. Stability of (*RS*)-[1-^{14}C]-2-PPA-CoA in HSA buffer solution was quantified by reverse phase HPLC using an

isocratic elution with 40% methanol in 0.05 M potassium phosphate buffer (pH 5.7) on a Microsorb-MV C_{18} column (150 x 4.6 mm, 5 μm, Rainin LC and Supplies, Walnut Creek, CA) at a flow rate of 1.0 mL/min and detected by UV absorbance (226 nm). Both HPLC methods allowed a good separation of 2-PPA-CoA from other interfering peaks, but not between (*R*)- and (*S*)-2PPA-CoA stereoisomers. (*R*)-, (*S*)- and (*RS*)-2PPA-CoA had the same retention time under the above HPLC conditions. Standard curves for 2-PPA-CoA in rat tissue homogenates and HSA were prepared by spiking synthetic (*R*)-2-PPA-CoA standards to rat tissues homogenate and HSA according to the procedures described above.

3.3 Results

3.3.1 Covalent Binding of (*RS*)-[1-^{14}C]-2-PPA-CoA to HSA

As shown in Chapter 2, 2-PPA-CoA is electrophilic and readily acylates GSH sulfhydryl to form the 2-PPA-SG conjugate. To determine whether a similar transacylation reaction could occur to protein nucleophiles, synthetic (*RS*)-[1-^{14}C]-2-PPA-CoA was incubated with HSA (a model protein) in phosphate buffer at physiological pH and temperature. Covalent binding of 2-PPA-CoA to HSA was evident after a 0.5-h incubation and could not be demonstrated in similar incubations performed with 2-PPA acid (Figure 3.3A). The extent of covalent binding of 2-PPA, via 2-PPA-CoA, to HSA was time-dependent and the rate of covalent adduct formation (100 pmol/mg HSA/h) was generally linear during the first 8 h of incubation (Figure 3.3A).

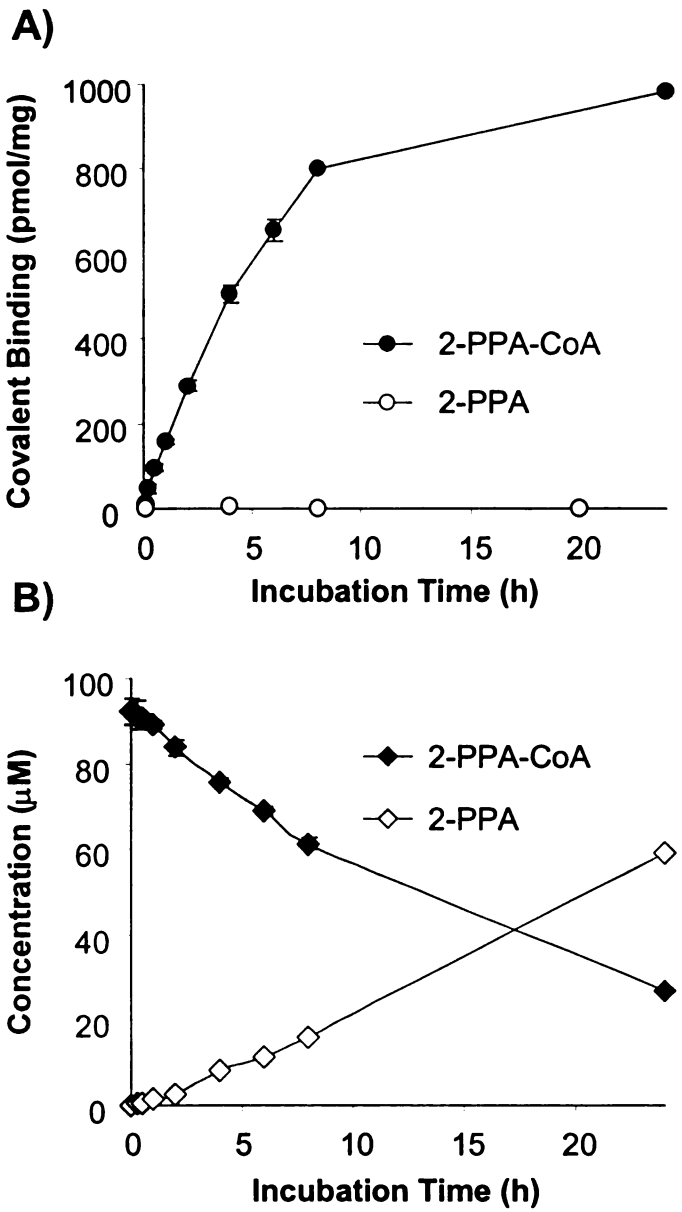


Figure 3.3 Time-dependent covalent binding (A) and chemical stability (B) of (*RS*)-[1-^{14}C]-2-PPA-CoA (0.1 mM) in 0.05 M potassium phosphate buffer containing HSA (30 mg/mL) at pH 7.4 and 37°C. Incubation with 0.1 mM (*RS*)-[1-^{14}C]-2-PPA (o) under the same conditions served as a negative control (A). Values represent means ± SD from triplicate incubations.

After a 24-h incubation, covalent adduct was detected at 970 pmol/mg HSA (0.064 mole/mole 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 (Figure 3.3B). HPLC analysis showed that the major degradation pathway was hydrolysis of the thioester bond of 2-PPA-CoA. Approximately 70% of 2-PPA-CoA was hydrolyzed to free 2-PPA after a 24-h incubation with HSA at 37°C (Figure 3.3B).

3.3.2 Characterization of 2-PPA-CoA Formation in Rat Liver Homogenate

Although three papers have reported the chiral inversion of 2-PPA, which implies formation of the 2-PPA-CoA intermediate (Yamaguchi and Nakamura, 1985; Nakamura and Yamaguchi, 1987; Yamaguchi and Nakamura, 1987), there is no direct evidence to show that 2-PPA forms 2-PPA-CoA in vitro or in vivo. Incubations of (*R*)-2-PPA with rat liver homogenate in the presence of cofactors of acyl-CoA formation led to the formation of 2-PPA-CoA that eluted on HPLC with a retention time identical to authentic 2-PPA-CoA standard (9.02 min, Figure 3.4B). Similar incubations without addition of ATP showed no peak eluting at the HPLC retention time of 2-PPA-CoA (Figure 3.4A). The identity of 2-PPA-CoA purified by HPLC from homogenate incubations was further confirmed using a Matrix-Assisted Laser Desorption Ionization (MALDI) time-of-flight instrument (PerSeptive Biosystems, Voyager Elite mass spectrometer, Framingham, MA) equipped with a nitrogen laser (337 nm). Fractions containing 2-PPA-CoA were collected in eluent and desalted through a C18 ziptip (Millipore Co, Bedford, MA). The eluent was mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 0.1% TFA, 50% ACN and 50% water (1:1) and the mixture (1 μL) was applied to a MALDI

sample plate. The MS showed two major ions at *m/z* 900 (100%) and 768 (25%), corresponding to MH^+ and $[CoASH + H]^+$, respectively.

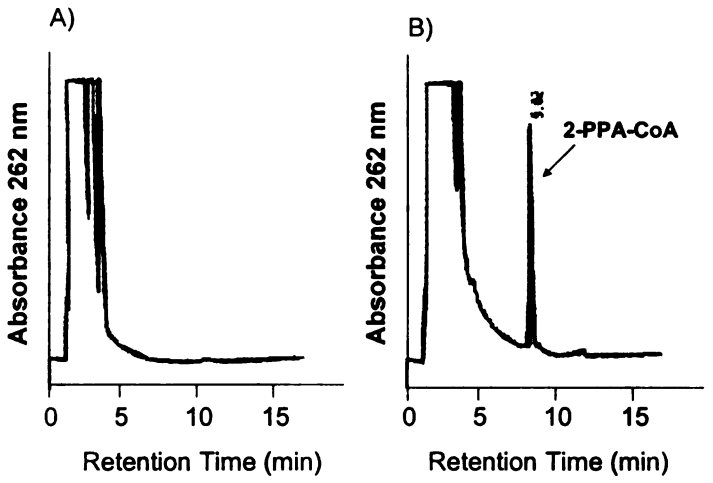


Figure 3.4 Representative reverse-phase HPLC chromatograms from 30 min incubations with rat liver homogenate in the absence (A) and presence (B) of ATP, a cofactor that is essential for acyl-CoA formation. Retention time of 2-PPA-CoA determined using authentic standard was 9.02 min.

The rate of 2-PPA-CoA formation in rat liver homogenate was also assessed over a concentration range of 0 to 4 mM (*R*)-2-PPA. Lineweaver-Burk plots of the data from pooled rat liver homogenate clearly demonstrate that more than one enzyme is involved in the catalysis of 2-PPA-CoA formation. The apparent K_{m1} and V_{max1} for the low affinity and high capacity enzyme were 1.9 ± 0.5 mM and 351 ± 44 pmol/min/mg protein (mean ± SD, N = 3), respectively (Figure 3.5). There were clear indications of a high affinity low capacity enzyme with an apparent K_{m2} and V_{max2} approximating 4.1 ± 0.2 μM and 16.8 ± 0.6 pmol/min/mg protein.

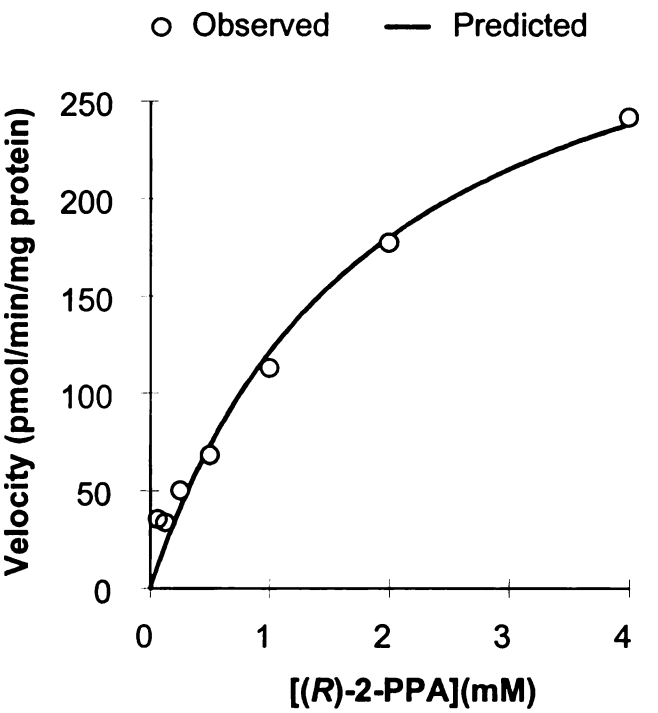


Figure 3.5 Kinetic analysis of the influence of (*R*)-2-PPA concentration on the rate of formation of 2-PPA-CoA by rat liver homogenate. Data are expressed as means ± SD (n = 3) with the line representing the best fit to the Michaelis-Menten equation.

3.3.3 Covalent Binding of (*RS*)-[1-^{14}C]-2-PPA to Rat Liver Homogenate

To examine if covalent binding of 2-PPA to liver proteins could be mediated via the acyl-CoA thioester formation pathway, we incubated (*RS*)-[1-^{14}C]-2-PPA with rat liver homogenate in the presence of cofactors that are essential for acyl-CoA formation. The reaction mixtures were then analyzed for 2-PPA-CoA formation and covalent binding. Formation of 2-PPA-CoA and covalent binding of 2-PPA to rat hepatic proteins

were detected when rat liver homogenate was incubated with CoA, ATP and Mg^{2+}, the cofactors for acyl-CoA formation. Both the formation and binding were dependent on ATP. Neither 2-PPA-CoA formation nor 2-PPA covalent adducts were detected in the absence of ATP (Figure 3.6). Covalent binding of 2-PPA to rat liver homogenate was time-dependent with an apparent plateau of 1000 pmol/mg protein being reached after 1 to 2 h of incubation, while 2-PPA-CoA achieved its plateau (5 nmol/mg protein) after 2 h of incubation (Figure 3.6).

To determine whether covalent adduct formation was associated with 2-PPA - CoA formation in tissue homogenate, inhibition studies were conducted with various fatty acids and xenobiotics known to form an acyl-CoA thioester themselves. Concentrations were chosen based on solubility considerations. Pretreatment with palmitic acid (0.1 mM) and ibuprofen (1 mM) led to an approximate 40% decrease in 2-PPA-CoA formation and covalent binding of 2-PPA to liver proteins (Figure 3.7). Under similar incubations, lauric acid (0.2 mM) showed a stronger inhibition of both covalent binding and 2-PPA-CoA formation by approximately 70% (Figure 3.7). It appears that the extent of covalent binding changes proportionally with the amount of 2-PPA-CoA formed in the incubations, and the ratio of covalent binding to 2-PPA-CoA formation was not significantly changed by these three inhibitors (Table 3.1). In contrast, octanoic acid (2 mM) decreased 2-PPA-CoA formation by approximately 90% but covalent binding only by 70%, resulting in a 3.4 fold increase in the ratio of protein covalent binding to 2-PPA-CoA formation (Table 3.1). In the absence of ATP, no 2-PPA-CoA was formed and little covalent binding was detected (Figure 3.7).

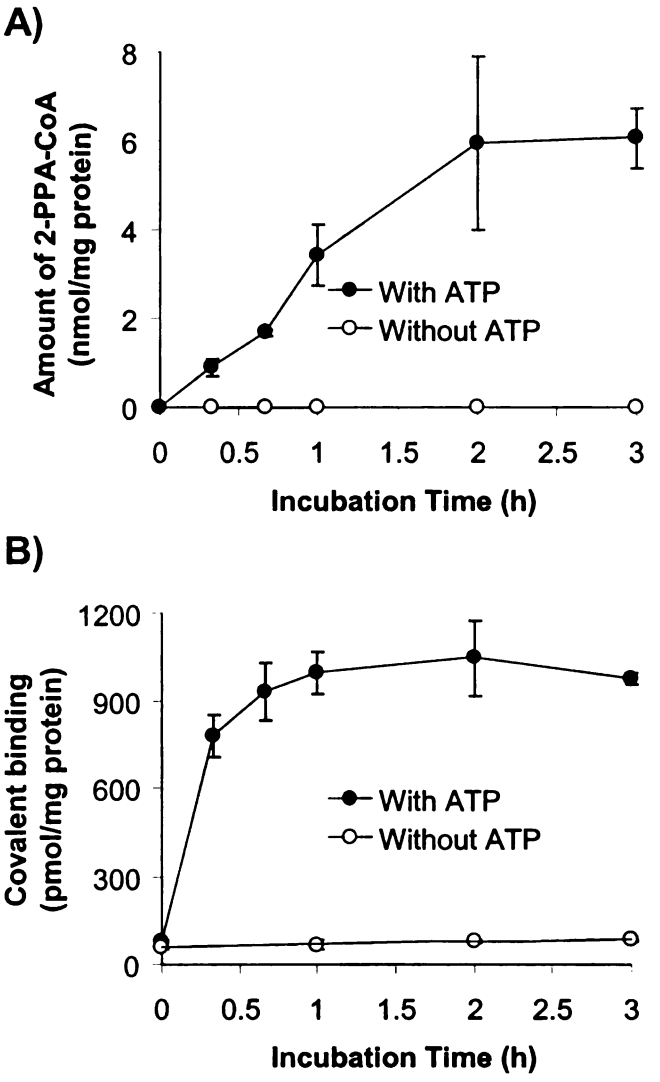


Figure 3.6 Time-dependent 2-PPA-CoA formation (A) and covalent binding of 2-PPA to hepatic proteins (B) in rat liver homogenate incubations with (*RS*)-[1-^{14}C]-2-PPA (1 mM), carried out in triplicate at 37°C in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM $MgCl_2$, 2.5 mM ATP and 1.5 mg/mL rat liver homogenate. Control incubations were conducted without the addition of ATP. Values represent means ± SD from triplicate incubations.

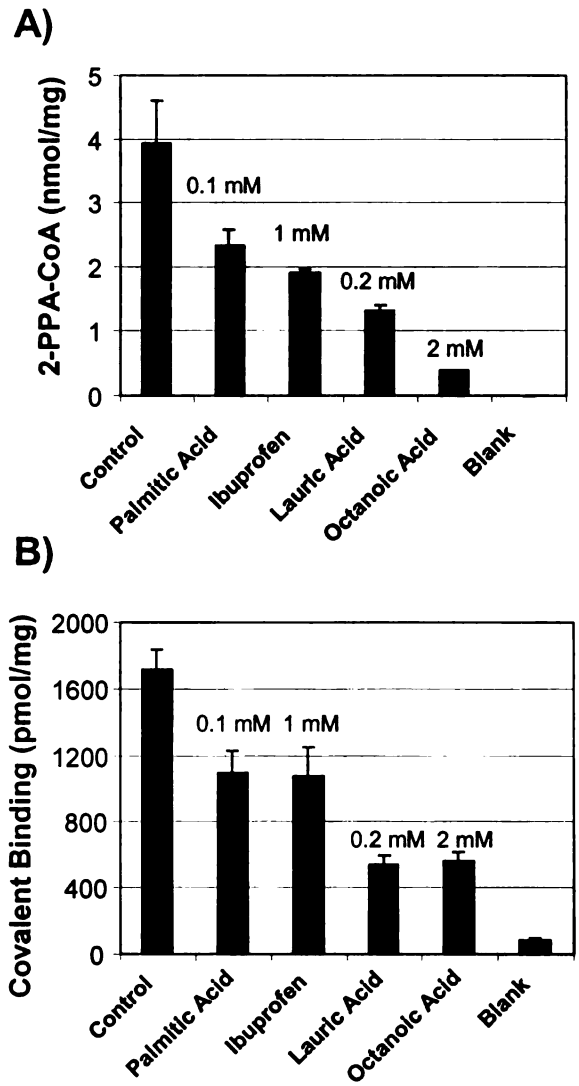


Figure 3.7 Effect of various acyl-CoA synthetase inhibitors on 2-PPA-CoA formation (A) and covalent binding of 2-PPA to hepatic proteins (B). Triplicate incubations were carried out for 3 h at 37°C in 150 mM Tris-HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM $MgCl_2$, 2.5 mM ATP, 1 mM (*RS*)-[1-^{14}C]-2-PPA and 1.5 mg/mL rat liver homogenate in the presence and absence of various inhibitors. The rat liver homogenate was pre-incubated with the inhibitor for 3 min. Blank incubations were conducted without the addition of ATP. Values represent means ± SD from triplicate incubations.

Table 3.1 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.

	2-PPA-CoA Formation (nmol/mg)	Covalent Binding (nmol/mg)	Covalent Binding to 2-PPA-CoA Ratio
Control[c]	3.92 ± 0.67	1.72 ± 0.12	0.44 ± 0.05
Palmitic Acid (0.1 mM)[d]	2.34[a] ± 0.26	1.09[b] ± 0.14	0.46 ± 0.03
Ibuprofen (1 mM)[d]	1.90[a] ± 0.07	1.07[a] ± 0.18	0.56 ± 0.11
Lauric Acid (0.2 mM)[d]	1.32[a] ± 0.09	0.54[b] ± 0.06	0.41 ± 0.03
Octanoic Acid (2 mM)[d]	0.38[a] ± 0.02	0.57[b] ± 0.05	1.48[b] ± 0.13

Results are shown as means ± SD of triplicate incubations in rat liver homogenate.
[a] $p < 0.05$ compared to corresponding control group, using t test
[b] $p < 0.01$ compared to corresponding control group, using t test
[c] Control incubations were carried out for 3 hrs 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 ATP, 1 mM (*RS*)-[1-^{14}C]-2-PPA and 1.5 mg/ml rat liver homogenate.
[d] The rat liver homogenate was pre-incubated with the inhibitor for 3 min.

3.4 Discussion

The withdrawal from the market of drugs containing carboxylic acid moieties, especially profen drugs such as benoxaprofen, suprofen and pirprofen, is disproportionally high relative to other classes of drugs. Idiosyncratic toxicity, such as liver damage, serious skin reactions and renal toxicity, sometimes associated with fever, rash and eosinophilia was the most frequent reason for their withdrawal (Zimmerman, 1994; Bakke et al., 1995; Boelsterli et al., 1995; van der Klauw et al., 1996). The mechanisms are not understood. In some cases, reactive drug metabolites cause the toxicity by binding covalently to tissue proteins (Boelsterli et al., 1995; Pumford et al., 1997). Acyl glucuronidation and acyl-CoA thioester formation are common metabolic pathways for most profen drugs, both of which generate of chemically reactive metabolites (Figure 3.1). Covalent binding of bilirubin acyl glucuronides to albumin was

demonstrated more than 20 years ago (McDonagh et al., 1984) and similar binding of several profen drugs to proteins has subsequently been observed (Spahn-Langguth et al., 1997; Li and Benet, 2002). In contrast, there are few reports on the chemical reactivity of profen acyl-CoA towards proteins. For this reason, we examined the covalent binding of 2-PPA-CoA to human serum albumin (HSA) and rat liver homogenate in vitro. 2-PPA was chosen because it is the simplest congener of profen drugs and forms 2-PPA-CoA, as inferred from the unidirectional chiral inversion of 2-PPA in vivo (Fournel and Caldwell, 1986).

Covalent binding of (*RS*)-[1-^{14}C]-2-PPA-CoA to HSA in phosphate buffer at pH 7.4 and 37°C was time-dependent. Approximately 970 pmol/mg HSA (0.064 mole/mole protein) covalent adduct was detected after 24 h of incubation, corresponding to 0.3% yield (Figure 3.3). We used racemic 2-PPA, since our recent studies demonstrated no stereoselectivity in the transacylation reaction of 2-PPA-CoA with GSH (Chapter 2 and Li et al., 2002). However, it is possible that the two diastereomers react differently with HSA even though no difference was detected with the much smaller GSH.

Hydrolysis of 2-PPA-CoA to 2-PPA free acid was accelerated by HSA. The disappearance of 2-PPA-CoA thioester in buffered HSA solution followed first-order kinetics with an apparent half-life of 13.4 h (Figure 3.3), but was negligible in the absence of HSA even after 24 h. The apparent half-life of 2-PPA-CoA in phosphate buffer (pH 7.4, 37°C) is approximately 12 days (Chapter 2 and Li et al., 2002). These findings are consistent with the well known esterase activity of albumin, also observed with acyl glucuronides, such as zomepirac and oxaprozin (Smith et al., 1985a; Wells et al., 1987).

The extent of covalent binding of 2-PPA-CoA to serum albumin seemed to depend on the species source of albumin, with human albumin forming more adduct than bovine (Chapter 2 and Li et al., 2002). 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, but was seen with HSA. Similar observations have been reported for acyl glucuronides. Tolmetin and zomepirac acyl glucuronides (Munafo et al., 1990; Smith et al., 1990) bound less covalently to BSA than to 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 (RSA) and human plasma proteins. Thus, small differences in albumin, such as animal origin and purity, can be important for the stability and covalent binding of chemical reactive species, including acyl-CoA thioesters and acyl glucuronides. Therefore, covalent binding data from various sources should be compared with caution. Nonetheless, regardless of the nature of the albumin preparation used, 2-PPA-CoA was shown to be reactive toward serum albumin in vitro, suggesting it's potential to bind covalently to other tissue proteins as well.

Acyl-CoA thioester derivatives do not cross plasma membranes and reach sites distant from where they are formed. The possibility of covalent binding of 2-PPA to HSA in vivo via the acyl-CoA pathway is relative low, since 2-PPA-CoA is not present in plasma, as opposed to the tissues where acyl-CoA thioester is formed. To examine the potential binding of acyl-CoA to tissue proteins, we conducted incubations with liver homogenate. Liver was selected because it is the major organ that metabolizes xenobiotic drugs, and severe drug-induced liver toxicity has been associated with the use of profen drugs.

Although previous studies have shown that 2-PPA can undergo unidirectional chiral inversion from (*R*)- to (*S*)-enantiomer in rats, which implied the formation of a 2-PPA-CoA intermediate, no direct evidence has been previously published to demonstrate the formation of 2-PPA-CoA in vivo or in vitro. Therefore, to demonstrate that tissue covalent adduct formation might be mediated by acyl-CoA intermediates, we characterized the ability of 2-PPA to form an acyl-CoA thioester in rat liver homogenate. A preliminary study showed that (*S*)-2-PPA (0.5 mM) formed no detectable 2-PPA-CoA thioester metabolites and further that (*S*)-2-PPA (0.5 mM) had little effect on the ability of (*R*)-2-PPA (0.5 mM) to form 2-PPA-CoA. Therefore, (*R*)-2-PPA was used in the kinetic studies and, because of the difficulty in obtaining enantiomerically pure [1-^{14}C]-2-PPA, (*RS*)-[1-^{14}C]-2-PPA (1.0 mM) was used in covalent binding studies.

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 co-elution with authentic 2-PPA-CoA and by mass spectrometry. No formation of 2-PPA-CoA was detected when ATP was absent (Figure 3.6A). Kinetic studies with (*R*)-2-PPA showed that more than one acyl-CoA synthetase might be involved in 2-PPA-CoA formation in tissue homogenate. 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. Preliminary studies showed that propionic acid (2 mM) had little effect on 2-PPA-CoA formation, indicating a lack of involvement of short-chain fatty acyl-CoA synthetase in this process.

Having demonstrated that 2-PPA was able to form 2-PPA-CoA in rat liver homogenate, we examined the covalent binding of 2-PPA to hepatic proteins via the acyl-CoA pathway in similar incubations. Our results demonstrate that protein covalent binding appears to depend on the formation of 2-PPA-CoA. 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 (Figure 3.6). When the formation of 2-PPA-CoA was inhibited, the extent of covalent binding decreased (Figure 3.7). For most inhibitors, both processes decreased proportionally, so that the ratio of covalent binding to 2-PPA-CoA formation remained unchanged (Table 3.1). This finding suggests that the 2-PPA-CoA derivative could be an important reactive metabolite, mediating covalent adduct formation in tissues.

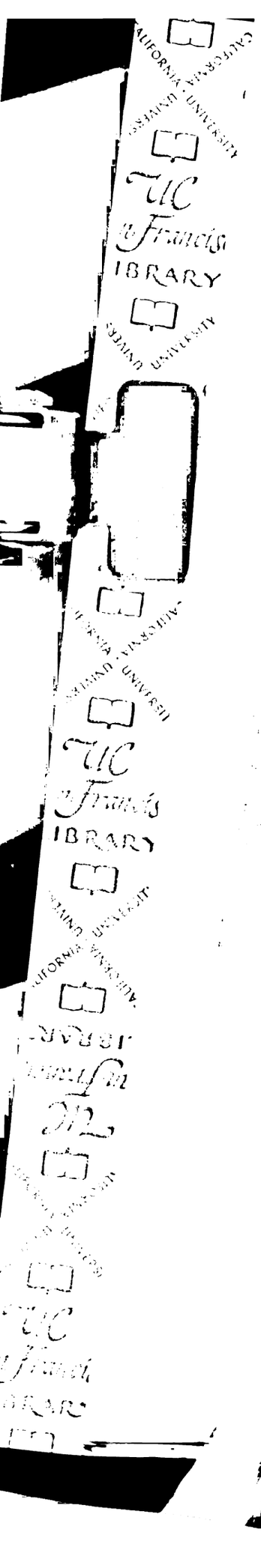
Time-dependent studies with 2-PPA in rat liver homogenate showed that 2-PPA readily reacts with nucleophilic sites on hepatic proteins. Covalent adduct formation quickly reached a plateau after 1 to 2 h incubation, even though a significant amount of 2-PPA-CoA thioester was still present in the incubation media (Figure 3.6). In fact, the concentration of 2-PPA-CoA in the incubation peaked and remained unchanged after 2 h of incubation. The reason is unclear. Possibly, 2-PPA covalent adducts in rat liver homogenate are unstable. Previous covalent binding studies with palmitoyl-CoA and nafenopin-CoA showed that acyl-CoA thioesters transacylated tissue proteins mainly via thioester bonds (Sallustio et al., 2000). Thioester bonds appear to be very stable in buffer, but not in rat liver homogenate. It has long been recognized that the liver has high

esterase activity, which may hydrolyze the thioester linkage between 2-PPA and hepatic proteins to release the 2-PPA free acid. Such protein thioesterase activity has been demonstrated for palmitoyl-protein thioesterases, which have recently been purified and cloned and shown to preferentially remove fatty acyl groups from proteins in vitro (Camp and Hofmann, 1993; Soyombo and Hofmann, 1997). Similar or the same protein thioesterases may exist in rat liver homogenate and remove 2-PPA from the 2-PPA-modified proteins. Therefore, the rapidly achieved plateau may reflect a steady state where the rate of adduct formation via the 2-PPA-CoA intermediate becomes equal to the rapid degradation rate of covalent adduct in the liver homogenate. This hypothesis is currently under investigation.

Consistent with the previous studies with nafenopin-CoA (Sallustio et al., 2000), palmitoyl-CoA did not inhibit covalent adduct formation by 2-PPA-CoA, despite inhibition of 2-PPA-CoA formation by palmitic acid. Similar results were also observed for two other acyl-CoA synthetase inhibitors, ibuprofen and lauric acid, but not with octanoic acid. Our results suggests that octanoyl-CoA may increase 2-PPA covalent adduct formation, as the ratio of covalent binding to 2-PPA-CoA formation was increased 3.4 fold. This observation could imply that 2-PPA-CoA and octanoyl-CoA have different protein targets or at least a different covalent binding site on the same protein. The unexpected observation that octanoyl-CoA appears to facilitate 2-PPA-CoA covalent binding to hepatic proteins is under further investigation.

The studies described here convince us that covalent binding of xenobiotic carboxylic acids to tissue proteins can be mediated by the acyl-CoA thioester pathway. However, this pathway may not be the major pathway for covalent binding of profen

drugs. As depicted in Figure 3.1, two alternative metabolic activation pathways of profen drugs, namely acyl glucuronidation and acyl-CoA formation, can lead to covalent adduct formation. Many recent in vivo studies with carboxylic acids attribute tissue covalent adduct formation to acyl glucuronidation; for example zomepirac, diflunisal, clofibric acid and valproic acid in drug-treated rats (Bailey and Dickinson, 1996) and diclofenac, sulindac and ibuprofen in drug-treated mice (Wade et al., 1997). However, clofibric acid, ibuprofen and valproic acid are able to form acyl-CoA thioesters, which may also potentially lead to covalent binding. Similarly, Hertz and Bar-Tana (1988) found that three hypolipidemic reagents, nafenopin, bezafibrate and MEDICA 16, were able to acylate membrane and cytosolic liver proteins in cultured rat hepatocytes. They attributed such selective protein binding to the formation of xenobiotic acyl-CoA thioesters. Since the authors did not give any evidence that protein acylation required the prior formation of xenobiotic acyl-CoA thioesters, they could not eliminate the possibility of the involvement of acyl glucuronides in the covalent binding (Hertz and Bar-Tana, 1988). In the cases listed above, the authors failed to distinguish the potential involvement of two metabolic activation pathways, namely acyl-CoA formation and acyl glucuronidation, in the adduct formation. The contribution of the two pathways in hepatocytes and rats is compared in Chapters 4 and 5.



Chapter 4

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

4.1 Introduction

2-Arylpropionic acids are a widely-used class of nonsteroidal anti-inflammatory drugs (NSAIDs). These compounds have a chiral center at the carbon alpha to the carboxylic acid, and only (*S*)-enantiomers exhibit significant anti-inflammatory activity (Shen, 1981). Nevertheless, 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 (Spahn-Langguth et al., 1997). 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 (Compernolle et al., 1977; Li and Benet, 2002). 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 originally for bilirubin (Etter-Kjelsaas and Kuenzle, 1975; McDonagh et al., 1984) and

subsequently for a large number of other compounds, including tolmetin (Munafo et al., 1993; Zia-Amirhosseini et al., 1994), zomepirac (Smith et al., 1986), carprofen (Iwakawa et al., 1988), diflunisal (McKinnon and Dickinson, 1989), fenoprofen (Volland et al., 1991) and ketoprofen (Dubois et al., 1993). In vitro studies also demonstrated that tolmetin acyl glucuronide (Ojingwa et al., 1994) and diclofenac acyl glucuronide (Kretz-Rommel and Boelsterli, 1994) could form 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 could cause drug-induced organ toxicity (Spahn-Langguth and Benet, 1992; Li and Benet, 2002).

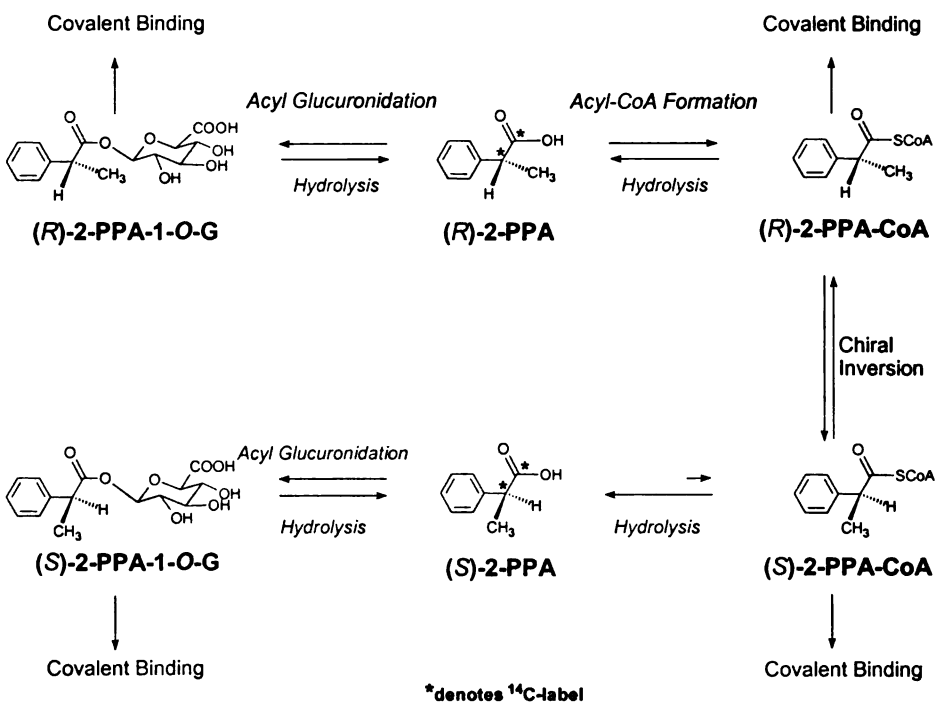


Figure 4.1 Scheme for the metabolic activation of (*R*)- and (*S*)-2-phenylpropionic acid by acyl-CoA formation and acyl glucuronidation.

Another remarkable feature of the metabolism of profen drugs is their unidirectional chiral inversion from the pharmacologically inactive (*R*)- to the active (*S*)-enantiomer. This has been documented by many in vivo studies using several species and 2-arylpropionic acid drugs (Mayer et al., 1994). The mechanism of the reaction is believed to involve initial enantioselective formation of acyl-coenzyme A (acyl-CoA) thioester followed by epimerization and finally hydrolysis to regenerate free acids (Figure 4.1). 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 (Hall and Quan, 1994).

In addition to mediating the chiral inversion of 2-arylpropionic acid drugs, activated acyl-CoA thioesters also are obligatory intermediates for the formation of amino acid conjugates, carnitine esters and hybrid triglycerides. Formation of thioester-linked acyl-CoA thioesters increases the electrophilicity of carboxylic acids. 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 (Bharadwaj and Bizzozero, 1995; Duncan and Gilman, 1996). In addition, covalent binding of nafenopin to human liver homogenate proteins requires prior formation of a nafenopin acyl-CoA thioester intermediate (Sallustio et al., 2000). Our recent studies (Chapter 2 and Li et al., 2002) have shown that the acyl-CoA of 2-phenylpropionic acid (2-PPA) is a better transacylation derivative than the 1-*O*-acyl glucuronide of 2-PPA in reactions with glutathione (GSH). 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. 2-PPA-*S*-acyl CoA (2-PPA-CoA) also reacted covalently with proteins, such as serum albumin and rat hepatic proteins (Chapter 3).

Acyl glucuronidation and acyl-CoA formation are common metabolic pathways for 2-arylpropionic acid drugs. Both pathways form reactive metabolites, which may lead to covalent binding of acidic drugs to proteins (Figure 4.1). The chemical reactivity of acyl glucuronides of acidic NSAIDs towards proteins has been extensively studied (Spahn-Langguth and Benet, 1992). In contrast, there is little information on the covalent binding of xenobiotic acyl-CoA thioesters. The relative importance of the two metabolic pathways for covalent binding is not known. The present studies were designed to examine their relative contribution to the covalent binding of 2-PPA (a model 2-arylpropionic acid) to protein in hepatocytes. If 2-PPA-CoA contributes more than 2-PPA-1-*O*-G, incubations with the (*R*)-isomer of 2-PPA should lead to greater covalent binding to protein than incubations with the (*S*)-isomer. By using two independent approaches, inhibition of 2-PPA acyl-CoA formation and acyl glucuronidation and subsequent effects on covalent binding, and 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.

4.2 Experimental Section

4.2.1 Chemicals

(*RS*)-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 (*RS*)-[1,2-$^{14}C_2$]-2-PPA, 2-PPA-CoA, and biosynthetic 2-PPA-1-*O*-G were obtained from previous studies in this thesis (Chapter 2 and Li et al., 2002). 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.

4.2.2 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 (*RS*)-[1,2-$^{14}C_2$]-2-PPA (0.1 mCi/mmol, dissolved in 1 mL of mobile phase) by reverse-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 x 150 mm, Chiral Technologies Inc., Exton, PA) at a flow rate of 0.8 mL/min and with UV detection at 226 nm. Fractions containing (*R*)- or (*S*)-[1,2-$^{14}C_2$]-2-PPA were combined and acetonitrile removed by evaporation under reduced pressure. The remaining aqueous solution (~50 mL) was acidified (pH 3.0) and extracted with ethyl ether (3 x 50 mL) until no significant radioactivity was detected in the aqueous phase. The combined ethyl ether extracts were evaporated under reduced pressure 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 co-elution on HPLC (as above) with commercially available (*R*)- and (*S*)-

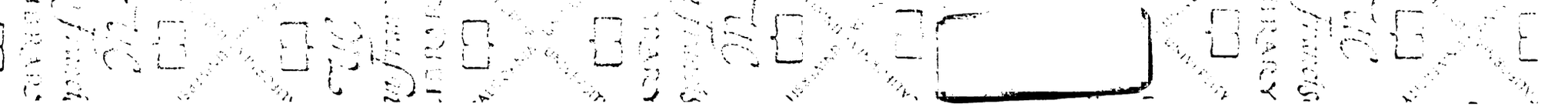
2-PPA standards (data not shown), and by ^{1}H-NMR analysis (which showed identical spectra for each enantiomer). ^{1}H NMR (deuterochloroform): δ 7.27-7.38 (m, 5H, phenyl ring), 3.74-3.80 (q, 1H, -CH-), 1.54-1.56 (d, 3H, $-CH_3$). The enantiomeric purities of (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were estimated, by HPLC, to be 86% and 96%, respectively. Both (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were more than 99% radiochemically pure as assessed by scintillation counting of HPLC fractions (0.1 mL) collected every 30 seconds as described above.

4.2.3 In Vitro Studies with Rat Hepatocytes

Freshly-isolated rat hepatocytes were prepared from one rat (300 g, male Sprague-Dawley) according to published procedures (Moldeus et al., 1978). Greater than 85% viability was achieved, as assessed by trypan blue exclusion. Incubations of hepatocytes (4 million cells/ml) with (*RS*)-, (*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 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 (*RS*)-[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 (*RS*)-[1,2-$^{14}C_2$]-2-PPA to the incubation mixture. Stock solutions of TMA, (*RS*)-, (*R*)- and (*S*)-[1,2-$^{14}C_2$]-2-PPA were 20 mM in water (pH = 7.0). Stock (-)-borneol solution was 100 mM in 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 over a 3 h period (as above).

After the addition of 2-PPA free acid to hepatocyte incubations, aliquots (3 mL) of the mixture were taken at indicated times and quenched with perchloric acid (7%, 2 mL) for determination of acyl-CoA and covalent binding to protein. The mixtures were centrifuged (1,000 *g*, 10 min) and the supernatants neutralized with 1 M NaOH and analyzed by reverse-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 5 times with 7% $HClO_4$/phosphate buffer, pH =7.4 (1:2, v/v), 8 times with ethanol/diethyl ether (3:1, v/v), and 8 times with MeOH/H_2O (4:1, v/v). The remaining protein pellets were heated in 1 M NaOH (0.65 mL) at 80°C for 1 h. Six hundred μL of the hydrolyzed protein solution was added to 600 μL of H_2SO_4 (1.5 M), followed by the addition of 10 mL of scintillation fluid (Hionic-Fluro, Packard BioScience Company, Meriden, CT), vortex mixed, and counted. Protein concentrations were determined in a 5 μL aliquot of the protein hydrolysate with a BCA protein assay reagent kit (Pierce, Rockford, IL) using bovine serum albumin as the standard, following the manufacturer's instructions. Covalent binding is expressed as pmol 2-PPA bound per mg of protein.

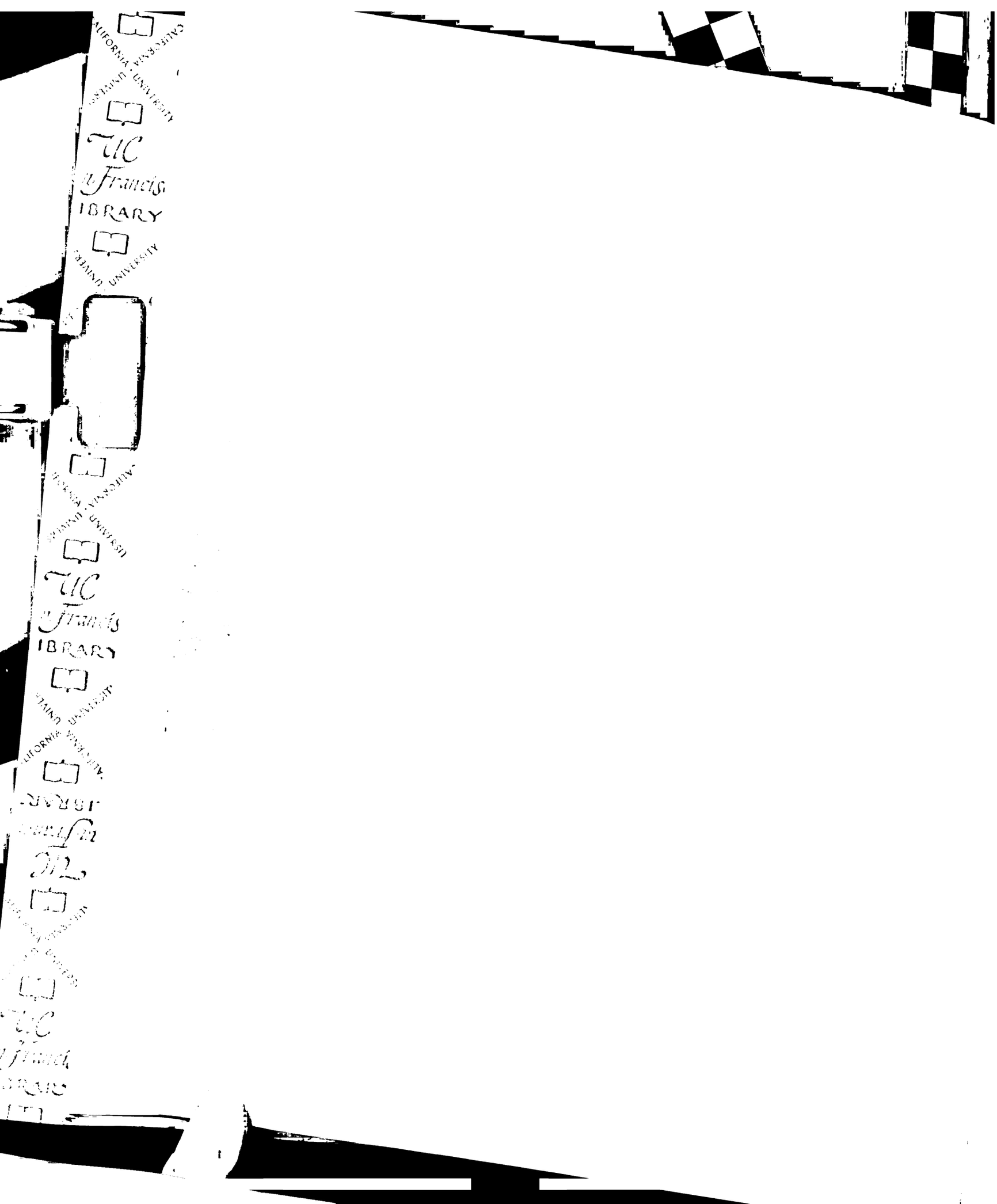


For the analysis of 2-PPA acyl glucuronidation in hepatocytes, 0.5 mL aliquots from incubation mixtures were taken at indicated time points and quenched with acetonitrile (0.5 mL) and HCl (1M, 12.5 μL). After centrifugation (10,000 *g*, 10 min), supernatants were analyzed by reverse-phase HPLC to quantify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

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

4.2.4 HPLC Analysis

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 C_8 Zorbax column (150 x 4.6 mm, 5μ, 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 C_{18} column (150 x 4.6 mm, 5μ, 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.



4.2.5 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 dried extracts using a Hewlett Packard HP 1100 LC/MSD bench-top 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 (*RS*)-2-PPA-1-*O*-G gave: *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 ($[C_6H_7O_6\text{-}CO_2\text{-}H_2O]^-$, 100%). Samples were analyzed by direct infusion into the ion source in the same mobile phase used for analysis of 2-PPA-CoA.

4.3 Results

4.3.1 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 reverse-phase HPLC of perchloric acid extracts with isocratic elution and UV-detection at 262 nM (the absorbance maximum for

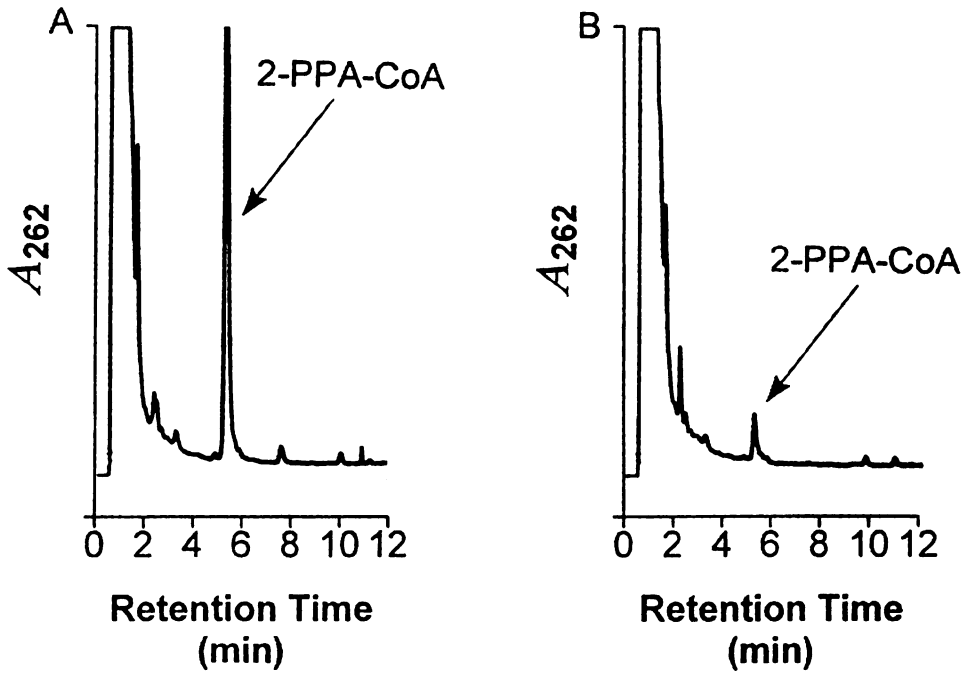


Figure 4.2 Representative reverse-phase HPLC analysis of 2-PPA-CoA from extracts of rat hepatocytes incubated with A) (*R*)-2-PPA or B) (*S*)-2-PPA.

CoA, Figure 4.2). Results showed that both (*R*)- and (*S*)-2-PPA form 2-PPA-CoA (Rt ~ 5.5 min) which co-eluted with synthetic (*RS*)-2-PPA-CoA standard (data not shown). This peak was absent from control. 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 extracts and comparison with a 2-PPA-CoA synthetic standard, which showed ions at *m/z* 291, *m/z* 330, *m/z* 393, *m/z* 410, *m/z* 428, *m/z* 491 and *m/z* 609 originating from the proposed cleavages shown in Figure 4.3.

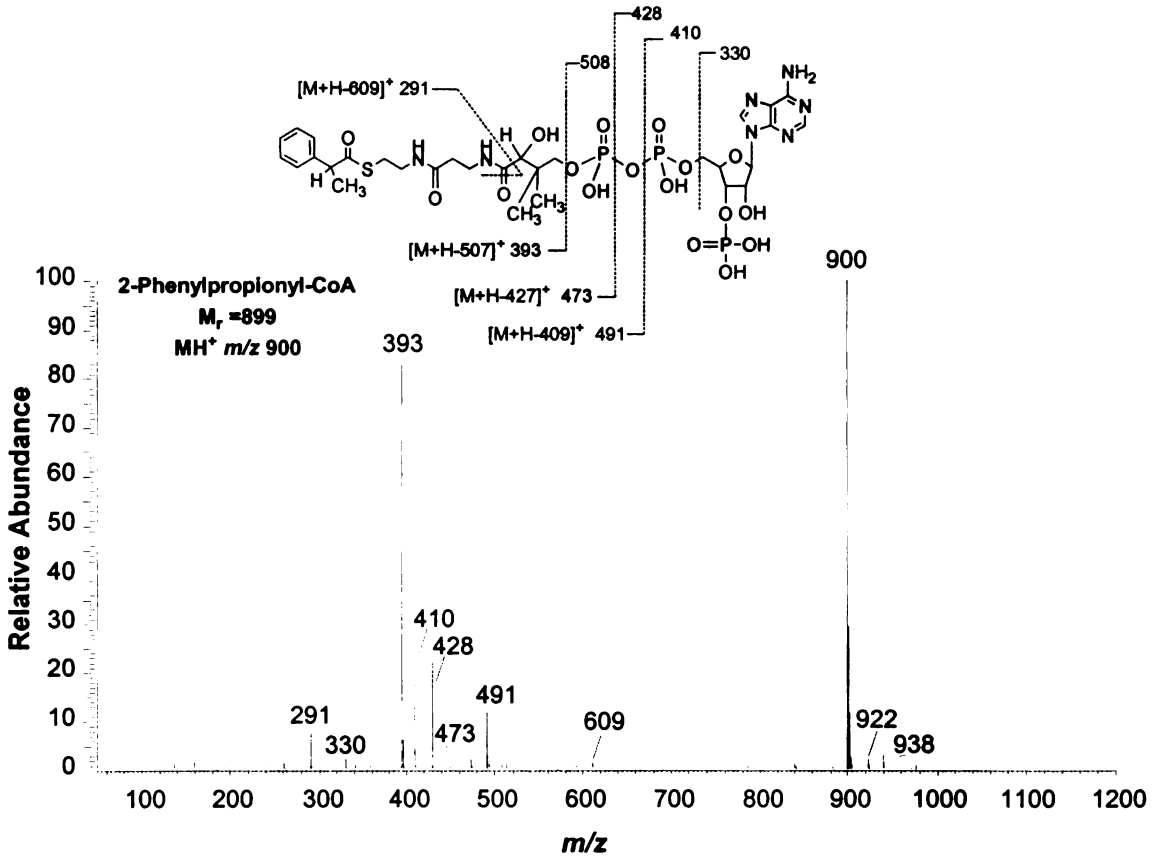


Figure 4.3 ESI/MS positive ion mass spectrum of 2-PPA-CoA isolated from the incubation of (*R*)-2-PPA (1 mM) with freshly isolated rat hepatocytes (4 million cells/mL) for 0.5 h. Suggested fragment ion are shown.

Analysis of 2-PPA acyl glucuronides formed during hepatocyte incubations in the presence and absence of 2-PPA (1 mM) was also performed by reverse-phase isocratic HPLC (Figure 4.4), which separated the (*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). Similar peaks were not detected in control hepatocyte incubation experiments. The identities of the resolved and purified (*R*)- and (*S*)-2-PPA-1-

O-G were confirmed by HPLC analysis of their β-glucuronidase and alkaline hydrolysis products using commercial (*R*)-2-PPA and (*S*)-2-PPA as reference standards. 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, *m/z* 59, *m/z* 105, *m/z* 113, *m/z* 149, *m/z* 175 and *m/z* 193 originating from the proposed cleavages shown in Figure 4.5.

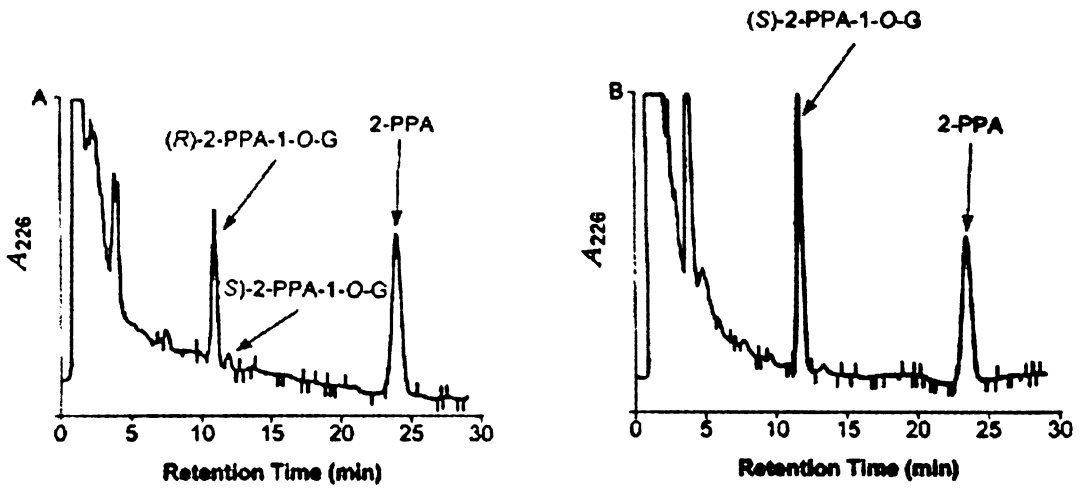


Figure 4.4 Representative reverse-phase HPLC analysis of 2-PPA-1-*O*-G from extracts of rat hepatocytes incubated with A) (*R*)-2-PPA or B) (*S*)-2-PPA.

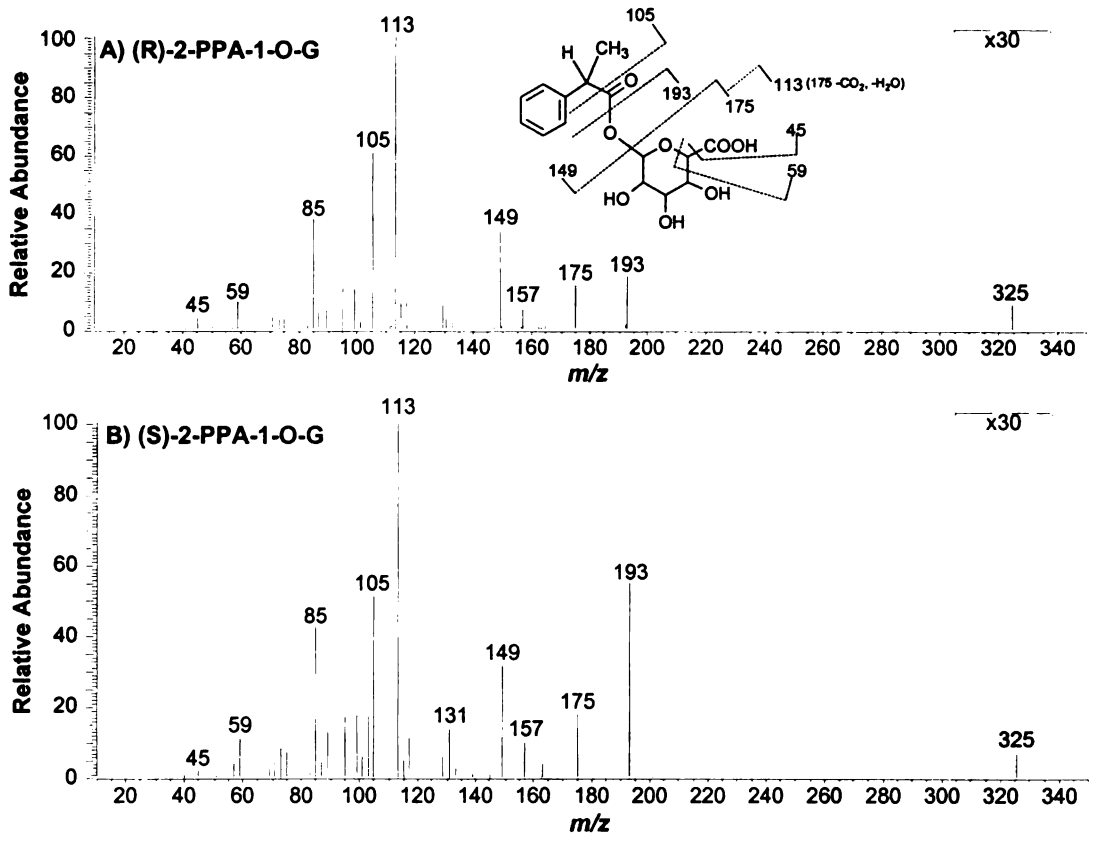
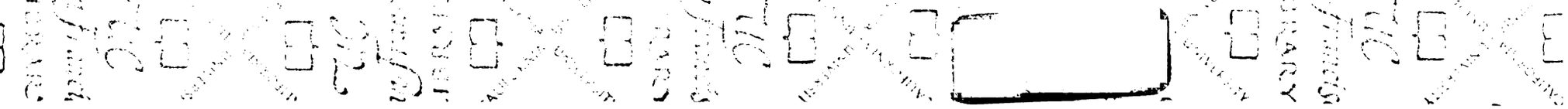


Figure 4.5 Spectra of product ions formed by CID of the $[M-H]^-$ ion (*m/z* 325) of 2-PPA-1-*O*-G obtained from extracts of rat hepatocytes incubated with A) (*R*)-2-PPA or B) (*S*)-2-PPA.

4.3.2 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 (*RS*)-$^{14}C_2$-2-PPA (1 mM), covalent binding to protein was time-dependent and reached a maximum plateau of nearly 230 pmol 2-PPA bound/mg protein in 1 h (Figure 4.6A). Formation of 2-PPA-CoA was rapid, reaching a maximum concentration of ~0.93 nmol/million cells in 15 min (Figure 4.6B), whereas 2-PPA acyl glucuronidation was roughly linear for 3 h, reaching



~40 nmol/million cells by the end of the incubation (Figure 4.6C). No 2-PPA-acyl glucuronide was detected by HPLC before 15 min. No covalent adduct formation, 2-PPA-CoA formation nor 2-PPA acyl glucuronidation was observed in incubations of heat-denatured hepatocytes treated with (*RS*)-$^{14}C_2$-2-PPA (1 mM) (data not shown). The maximum concentration of 2-PPA acyl glucuronide measured (3 h time-point) was nearly 40-fold higher than that of 2-PPA-CoA, which is consistent with the relatively higher capacity of acyl glucuronidation.

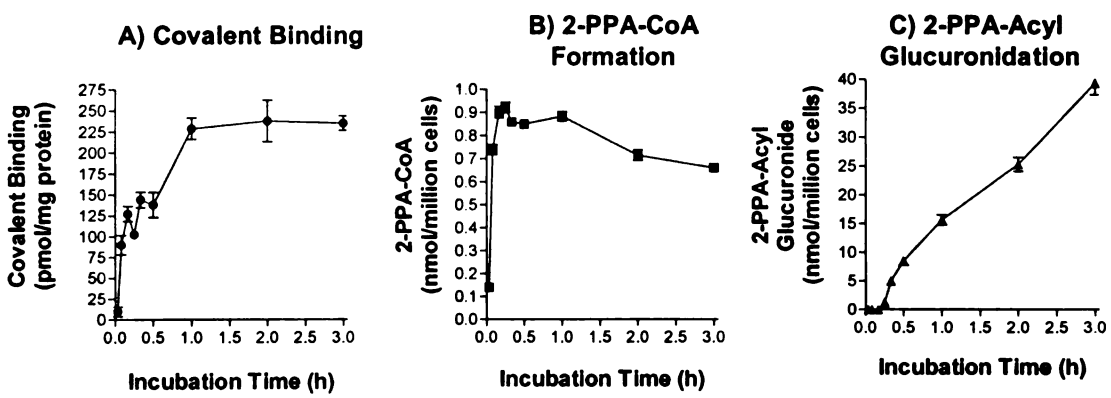


Figure 4.6 Time-course of the A) covalent binding, B) acyl-CoA formation and C) acyl glucuronidation of (*RS*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) incubated with freshly-isolated rat hepatocytes (4 million cells/mL). Values are expressed as the mean ± SD of triplicate incubations.

4.3.3 Inhibition Studies

To determine the relative importance of 2-PPA acyl glucuronidation and acyl-CoA formation in covalent binding of 2-PPA to proteins in hepatocytes, we examined the effect of enzyme inhibitors (Figure 4.7). Experiments were performed with (*RS*)-$^{14}C_2$-2-PPA (1 mM) in the presence or absence of TMA (2 mM) or (-)-borneol (1 mM) to inhibit 2-PPA-CoA formation and 2-PPA acyl glucuronidation, respectively. As shown in Figure 4.7, the covalent binding of 2-PPA to protein decreased by 53% in hepatocytes treated with TMA, which is similar to the 66% decrease in 2-PPA-CoA formation. No significant effect of TMA was observed on 2-PPA acyl glucuronidation. Conversely, treatment with (-)-borneol, which completely inhibited formation of 2-PPA acyl glucuronide, only decreased covalent binding by 18.7% after 3 h of incubation.

4.3.4 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 4.8). Covalent binding of 2-PPA to hepatocyte proteins was 4.5-fold greater for the (*R*)-2-PPA isomer (567 pmol/mg protein) than for the (*S*)-2-PPA isomer (125 pmol/mg protein) after 3 hours of incubation (Figure 4.8A). 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 ($R/S = 0.67$).

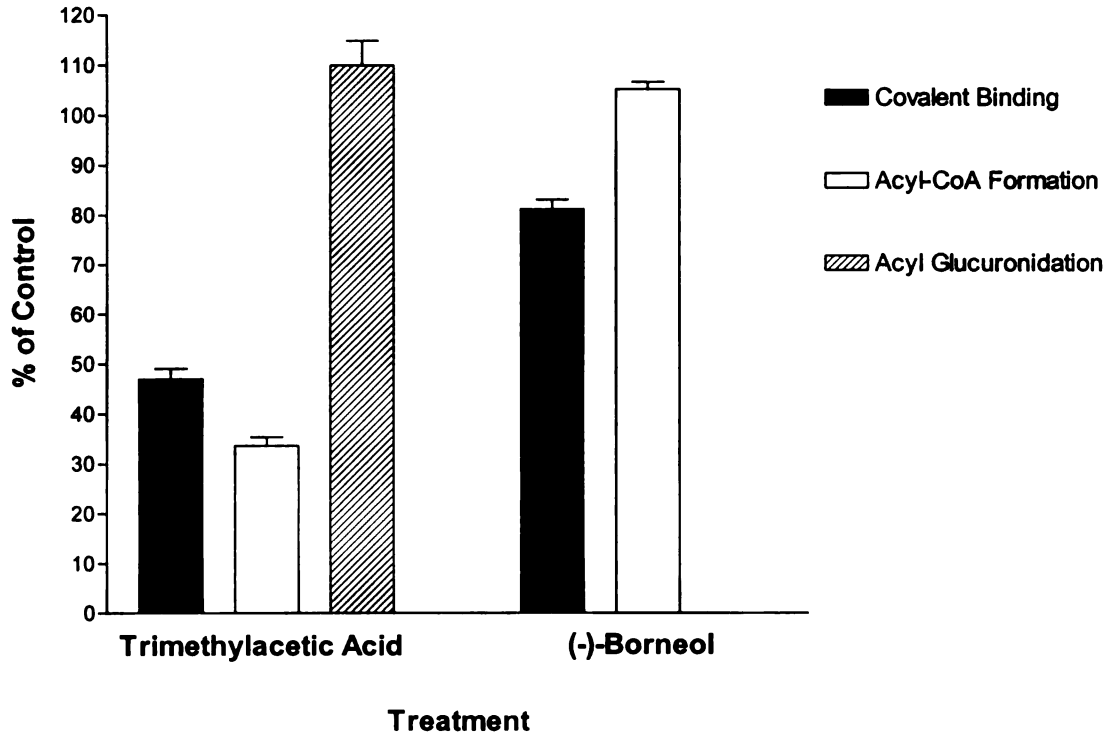


Figure 4.7 Effect of trimethylacetic acid (2 mM) and (-)-borneol (1 mM) on the covalent binding, acyl-CoA formation and acyl-glucuronidation of (*RS*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) in incubations with freshly isolated rat hepatocytes (4 million cells/mL) after 3 h of incubation. Values are expressed as the mean ± SD of triplicate incubations.

4.4 Discussion

The withdrawal from the market of drugs containing a carboxylic acid moiety, especially 2-arylpropionic acid-type drugs, is disproportionally high relative to other classes of drugs. The most frequent cause for withdrawal was idiosyncratic toxicity, such as liver damage, serious skin reactions and renal toxicity, sometimes associated with

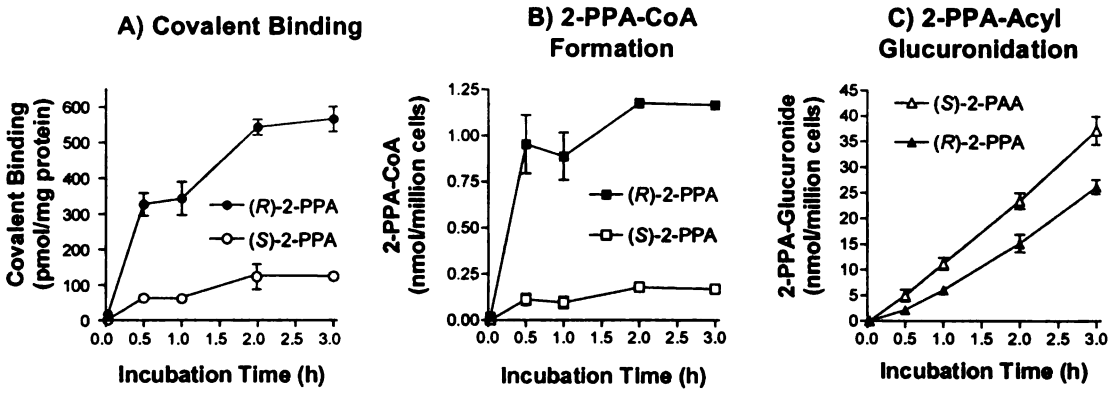


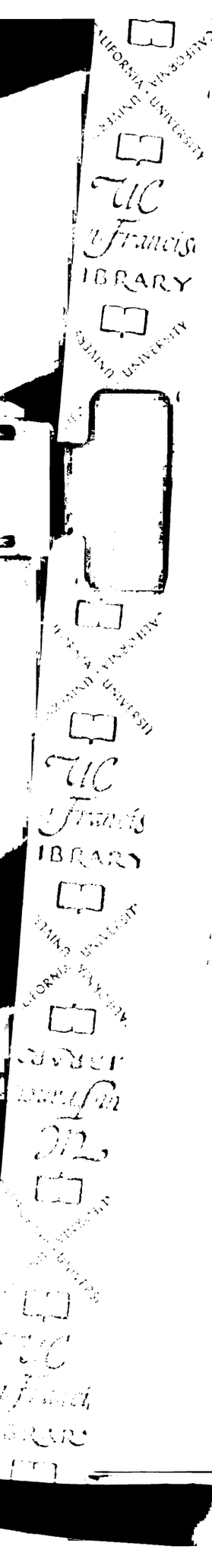
Figure 4.8 Time-course of A) covalent binding, B) acyl-CoA formation and C) acyl glucuronidation of (*R*)-[1,2-$^{14}C_2$]-2-PPA or (*S*)-[1,2-$^{14}C_2$]-2-PPA (1 mM) incubated with freshly-isolated rat hepatocytes (4 million cells/mL). Values are expressed as the mean ± SD of triplicate incubations.

fever, rash and eosinophilia (Zimmerman, 1994; Bakke et al., 1995). The mechanisms responsible for these side-effects are poorly understood. Covalent modification of cellular proteins by chemically-reactive species formed during the metabolism of the drugs has been suggested as a possibility. For acidic drugs, acyl glucuronidation and acyl-CoA thioester formation are common metabolic pathways, which both lead to the reactive metabolites (Figure 4.1). 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 (Spahn-Langguth et al., 1997) following its discovery for bilirubin (Kuenzle et al., 1966; McDonagh et al., 1984). In contrast, few reports in the literature concern the chemical reactivity of xenobiotic acyl-CoA conjugates towards proteins. Sallustio et al. (2000) showed that nafenopin-CoA bound covalently to proteins in tissue homogenate. The present studies provide direct evidence that acyl-CoA thioesters of 2-arylpropionic acids can bind covalently to hepatocyte proteins, and to a greater extent than acyl glucuronides.

2-PPA was chosen as a simple model compound. 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 detected (Dixon et al., 1977). Unidirectional chiral inversion observed in vivo results from the formation of 2-PPA-CoA (Fournel and Caldwell, 1986). Thus, 2-PPA was selected here as a model compound to investigate the relative reactivity of acyl glucuronides and acyl-CoA thioesters with hepatocyte proteins.

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 4.6). Covalent adduct formation was undetectable in heat-denatured hepatocytes, indicating that viable cells and functional enzymes are necessary for metabolic activation of 2-PPA. Selective inhibition of each metabolic pathway led to a decrease in covalent adduct formation (Figure 4.7), indicating that both pathways contribute to covalent binding. 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% (Figure 4.7). 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 nearly 40-fold greater than 2-PPA-CoA formation (Figure 4.6). 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 towards proteins. A higher contribution of the acyl-CoA pathway to covalent binding therefore may result from the higher chemical reactivity of acyl-CoA thioesters towards proteins, compared to acyl glucuronides. Recently, we showed, in vitro in buffer (0.05 M potassium phosphate, pH 7.4), that the reactivity of GSH (a model nucleophile) with 2-PPA-SCoA is 70 times greater than with 2-PPA-1-*O*-G, which undergoes acyl migration to less reactive isomers (Chapter 2 and Li et al., 2002).

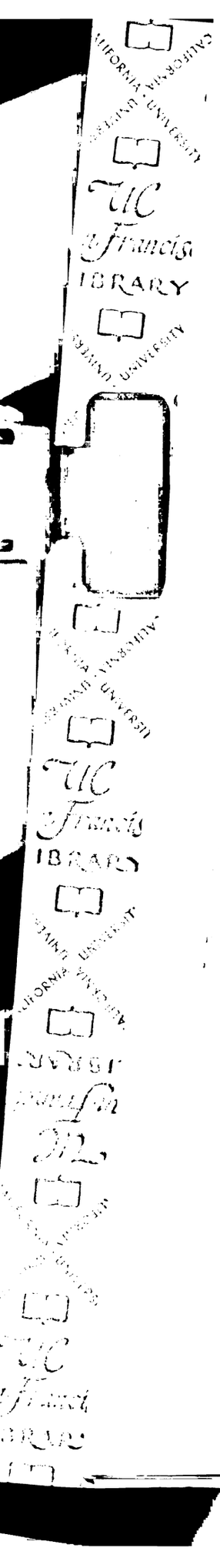
2-PPA undergoes chiral inversion from the (*R*)- to the (*S*)-enantiomer via an acyl-CoA thioester intermediate (Fournel and Caldwell, 1986). Formation of 2-PPA-CoA thioester is enantioselective for the (*R*)-enantiomer (Figure 4.8B), whereas, acyl glucuronidation is selective for the (*S*)-2-PPA enantiomer (Figure 4.8C). Both metabolic activation pathways could cause covalent binding of the drug. We surmised that 2-PPA-protein adduct formation would be greater with the (*R*)- than the (*S*)-enantiomer if bioactivation by 2-PPA-CoA formation is important. Conversely, (*S*)-2-PPA isomer should form more covalent adducts, if acyl glucuronidation is the most important. The hepatocyte studies with (*R*)- and (*S*)-2-PPA showed that covalent binding is

enantioselective for the (*R*)-enantiomer. Covalent binding was 4.5 fold greater after 3 h of incubation with (*R*)-2-PPA than with (*S*)-2-PPA (Figure 4.8A). 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 (Figure 4.7).

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 (Benet et al., 1993). These studies strongly indicate the potential involvement of acyl glucuronides in the covalent binding of acidic drugs to plasma protein in vivo, which may explain their allergic effects (Zia-Amirhosseini et al., 1995). Conversely, acyl-CoA thioester derivatives cannot cross plasma membranes and reach sites distant from where they are formed. It is very unlikely that acyl-CoA thioesters mediate the covalent adduct formation of acidic drugs in plasma, since they are not present. However, both metabolic activation pathways, namely acyl glucuronidation and acyl-CoA formation, might cause covalent binding of acidic drugs to hepatic proteins and therefore contribute to drug-induced organ toxicity. In vitro tissue homogenate studies with tolmetin (Ojingwa et al., 1994) and diclofenac (Hargus et al., 1994; Kretz-Rommel and Boelsterli, 1994) showed

that acyl glucuronides could contribute to the formation of hepatic protein adducts. Recent studies with nafenopin also showed that its covalent binding to liver proteins in vitro is directly associated with acyl-CoA formation (Sallustio et al., 2000). Therefore, acyl glucuronide formation is not the only mechanism for covalent binding of acidic drugs to proteins in vivo and the possible involvement of acyl-CoA formation should always be considered too, which has not always been done (Bailey and Dickinson, 1996; Wade et al., 1997).

A possible explanation for the increased covalent binding of the (*R*)-2-PPA isomer to hepatocyte protein relative to the (*S*)-antipode may be the greater tendency of (*R*)-2-PPA-1-*O*-acyl glucuronide to undergo acyl migration (Akira et al., 2000; Li et al., 2002) to isomers that are able to form Schiff-base-type protein adducts. However, since the complete inhibition of 2-PPA acyl glucuronidation in rat hepatocyte experiments (Figure 4.7) 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 (amides or imines) were formed via glucuronidation. In addition, studies have shown that when a racemic dose of a 2-arylpropionic acid NSAID, fenoprofen (Volland et al., 1991) or benoxaprofen (Dahms and Spahn-Langguth, 1996), is given to volunteers, there is a slight preponderance of protein adduct in blood with the (*S*)-isomers. Those studies concluded that because of (*R*) to (*S*) stereoinversion, acyl glucuronide concentrations in plasma were greater for the (*S*)- than for the (*R*)-isomers, leading to increased (*S*)-isomer adduct formation. We propose that increased 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 has 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 in vitro than 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 that could contribute to the hepatotoxicity of this class of drugs.

Chapter 5

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

5.1 Introduction

At least two metabolic pathways, acyl glucuronidation and acyl-CoA formation, generate reactive metabolites of profen drugs (Boelsterli, 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, undergoing nucleophilic displacement reactions with: (1) free hydroxyl anions in buffer, resulting in hydrolysis to the parent aglycone; (2) hydroxyl groups on the glucuronic acid ring, resulting in intramolecular acyl migration to β-glucuronidase-resistant isomers (Compernolle et al., 1977; Li and Benet, 2002); and (3) glutathione (GSH), resulting in the formation of *S*-acyl glutathione conjugates (Shore et al., 1995; Li et al., 2002; Olsen et al., 2002). They also react with proteins in vitro and in vivo, as first shown for bilirubin glucuronides (Etter-Kjelsaas and Kuenzle, 1975) and later for profen glucuronides (Smith et al., 1986; 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 are also intermediates for the formation of amino acid conjugates (Hutt and Caldwell, 1990), acyl carnitine and choline derivatives (Sastry et al., 1997), as well as hybrid triacylglycerides (Fears, 1985; Williams et al., 1986; Sallustio et al., 1988). All these processes require a reactive thioester bond, and thus acyl-CoA derivatives are believed to be electrophilic. 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 non-enzymatically 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 demonstrate that the acyl-CoA thioester derivative of 2-phenylpropionic acid (2-PPA) is able to acylate GSH sulfhydryl to form 2-PPA-*S*-acyl glutathione (2-PPA-SG) approximately 70 times faster than similar reactions with 2-PPA-1-*O*-acyl glucuronides (2-PPA-1-*O*-G) (Chapter 2 and Li et al., 2002). 2-PPA-*S*-acyl CoA (2-PPA-CoA) was also shown to bind irreversibly to proteins, such as serum albumin (Chapter 2 and Li et al., 2002) and hepatic proteins (Chapter 4).

Most profen drugs are metabolized in vivo to acyl glucuronides and acyl-CoA thioesters, which are reactive and believed to form covalent adducts with proteins in vivo. The present studies were designed to quantitate the contribution of 2-PPA acyl

glucuronidation and acyl-CoA formation to protein covalent adduct formation in vivo in rats. The results reported here strongly suggest that metabolic activation to 2-PPA-CoA contributes more to formation of protein adducts in the liver than metabolic activation to the acyl glucuronide.

5.2 Experimental Section

5.2.1 Materials

(*RS*)-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 (Fair Lawn, NJ), corn oil and trifluoroacetic acid (TFA) from Sigma Co. (St. Louis, MO). Hionic-Fluor scintillation fluid was purchased from Packard BioScience Co. (Meriden, CT). *(RS)*-[1-^{14}C]-2-PPA was synthesized by American Radiolabeled Chemicals, Inc (St. Louis, MO). Synthetic 2-PPA-CoA and the biosynthetic 2-PPA-1-*O*-G were available from previous studies in this laboratory (Chapter 2 and Li et al., 2002). TMA-*S*-acyl CoA (TMA-CoA) was synthesized by conventional procedures employing chloroformate, as we reported previously (Chapter 2 and Li et al., 2002). All solvents used for HPLC analysis were of chromatographic grade.

5.2.2 Animals

Male Sprague-Dawley rats (250-300 g) were purchased from Bantan & Kingman Universal (Livermore, CA), maintained in a controlled housing environment with 12 h light/dark cycles, and received standard laboratory chow and water ad libitum. Rats were

allowed at least 3 days to become acclimated to the housing conditions before use in experiments. All animal use was approved by the University of California San Francisco Committee on Animal Research.

5.2.3 In Vivo Inhibition Studies with (*RS*)-2-PPA

To determine the optimal inhibitory doses of (-)-borneol and TMA, dose-dependent inhibitory effects on 2-PPA acyl glucuronidation and acyl-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 min and 30 min); 3) (-)-borneol (160 mg/kg – 480 mg/kg in corn oil, 1 ml/rat, 30 min); 4) trimethylacetic acid (TMA, 400 mg/kg – 800 mg/kg in corn oil, 1 ml/rat, 10 min), before an i.p. injection of 2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Rats were decapitated 2 h after 2-PPA administration, their livers were removed and immediately frozen in liquid nitrogen. In these preliminary studies, one rat was utilized for each dosage treatment. Pretreatment with TMA 10 min or 30 min before 2-PPA dosing had approximately the same inhibitory effect on the metabolic activation of 2-PPA; 30 min was used in later studies.

Rats were pretreated i.p. with corn oil (control, 1ml/rat) , (-)-borneol (320 mg/kg in corn oil, 1 ml/rat) or TMA (500 mg/kg in corn oil, 1 ml/rat) 30 min before receiving an injection of *(RS)*-2-PPA (130 mg/kg in 0.9% saline, i.p., 0.5 ml/rat). Rats were then decapitated at 0, 0.25, 0.5, 1 and 2 h and their livers removed, frozen and stored at -80°C. Two rats were used for each time point for control, (-)-borneol and TMA pretreatment groups.

An additional 12 rats (4 each for each treatment) received (*RS*)-[1-^{14}C]-2-PPA (0.1 mCi/mmol, 130 mg/kg in 0.9% saline, 0.5 ml/rat) 30 min after pretreatment with corn oil (control, 1ml/rat), (-)-borneol (320 mg/kg in corn oil, 1 ml/rat) or TMA (500 mg/kg in corn oil, 1 ml/rat) and were killed 2 h later. Livers were stored at –80°C.

5.2.4 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 by 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 homogenate was immediately denatured with 0.75 ml $HClO_4$ (7%), mixed vigorously and centrifuged (10,000 *g*, 10 min). Supernatants were neutralized with 1M NaOH and analyzed by reverse-phase HPLC as described in Chapter 4. Protein pellets from the livers of (*RS*)-[1-^{14}C]-2-PPA-treated rats were used to determine adduct formation.

5.2.5 Analysis of 2-PPA Acyl Glucuronides in Livers of 2-PPA-Treated Rats

For the analysis of 2-PPA acyl glucuronidation in rat livers, frozen rat liver (0.5 g) was homogenized in 0.5 ml of potassium phosphate buffer (0.05 M, pH 5). The homogenate was immediately denatured by the addition of 0.5 ml of acetonitrile (ACN) and centrifuged at 10,000 *g* for 10 min. The supernatant was analyzed by HPLC as described in Chapter 4.

5.2.6 Irreversible Binding of (*RS*)-[1-^{14}C]-2-PPA to Rat Liver Proteins

Irreversible binding of 2-PPA to proteins was measured by scintillation counting of exhaustively washed hepatic protein precipitates as described previously (Chapter 4).

5.2.7 Statistical Analysis

ANOVA 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 $p < 0.05$.

5.3 Results

5.3.1 HPLC Analysis

Formation of 2-PPA-CoA in rat liver was monitored by isocratic reverse-phase HPLC with UV-detection at 262 nm, the absorbance maximum for CoA (Figure 5.1). The thioester, which coeluted with synthetic standard, was detected in 2-PPA-treated but not in saline-treated rats. Pretreatment with (-)-borneol had little effect on 2-PPA-CoA formation (Figure 5.1C vs 1A), but the 2-PPA-CoA peak was markedly decreased by TMA pretreatment (Figure 5.1B). TMA-CoA, which coeluted with synthetic standard, was also detected in TMA-pretreated rats (Figure 5.1B).

Analysis of 2-PPA acyl glucuronides in livers from 2-PPA-treated rats was also performed by reverse-phase isocratic HPLC (Figure 5.2), which separated the (*R*)- and

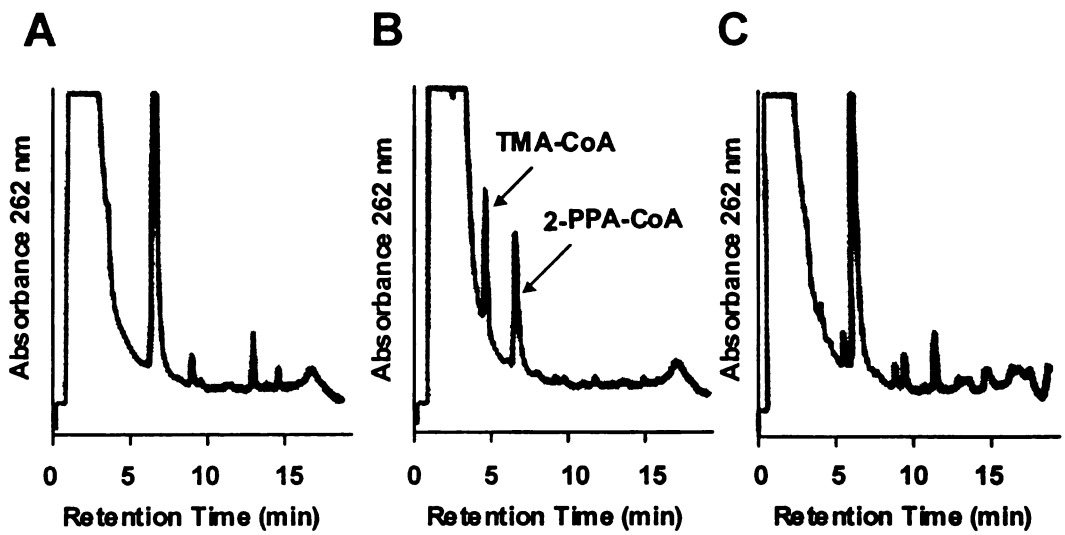


Figure 5.1 Representative reverse-phase HPLC analysis of 2-PPA-CoA in liver extracts from rats dosed with (*RS*)-2-PPA. Rats were pretreated i.p. with A) corn oil (control, 1ml/rat), B) TMA (500 mg/kg in corn oil, 1 ml/rat) or C) (-)-borneol (320 mg/kg in corn oil, 1 ml/rat) 30 min before receiving an injection of (*RS*)-2-PPA (130 mg/kg in 0.9% saline, i.p., 0.5 ml/rat). Rats were killed 2 h after 2-PPA administration and their livers collected and analyzed by HPLC.

(*S*)-2-PPA-1-*O*-G diastereomers, as well as 2-PPA acyl glucuronide migration isomers (data not shown). 2-PPA acyl glucuronides were not detected in liver extracts from saline-treated controls. Pretreatment with TMA decreased the total formation of 2-PPA acyl glucuronides. The *R/S* ratio of 2-PPA acyl glucuronides was increased in TMA-

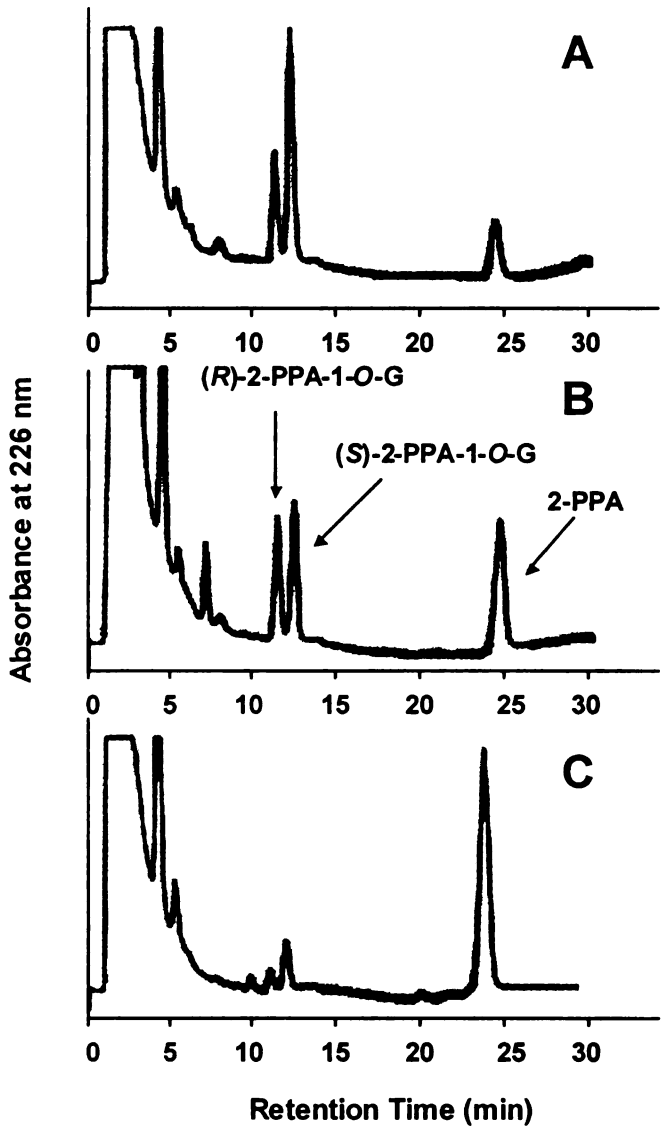


Figure 5.2 HPLC analysis of 2-PPA acyl glucuronides in liver extracts from rats dosed with (*RS*)-2-PPA. Rats were treated as in Figure 5.1.

pretreated rats by selectively increasing (*R*)-2-PPA-1-*O*-G levels and decreasing (*S*)-2-PPA-1-*O*-G levels (Figure 5.2B), which is consistent with the inhibitory effect of TMA on 2-PPA-CoA formation, the key step in chiral inversion of 2-PPA from (*R*)- to (*S*)-isomers. Pretreatment with (-)-borneol significantly decreased 2-PPA acyl

glucuronidation (Figure 5.2C). Since (-)-borneol and TMA have low absorbance at 226 nm, glucuronide conjugates of (-)-borneol and TMA did not appear.

To minimize degradation of 2-PPA-CoA and 2-PPA-1-*O*-G during homogenization, we homogenized under acidic conditions (pH 5), on ice and quickly (< 40 sec). The homogenate was denatured immediately to minimize enzymatic degradation of 2-PPA-CoA and 2-PPA-1-*O*-G. Tissues were kept on dry ice and HPLC analysis 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 the extraction conditions.

5.3.2 Optimal Dose of Inhibitors

(-)-Borneol inhibition studies were done as described previously (Watkins and Klaassen, 1982; Hong et al., 1999) with minor modifications. Preliminary studies showed that a high dose of (-)-borneol (750 mg/kg in corn oil) (Watkins and Klaassen, 1982; Watkins and Klaassen, 1983), causes acute CNS effects in rats. Rats appeared confused and dizzy and activity was markedly reduced 10 min after (-)-borneol administration. The toxic symptoms were transient and the (-)-borneol-treated rats appeared to completely recover 1 h after dosing. To avoid these effects, we ran dose-dependent inhibition studies (0, 160, 320 and 480 mg/kg) to determine the lowest effective inhibitory dose. 2-PPA acyl glucuronidation was completely inhibited by 320 and 480 mg/kg doses of (-)-borneol, without obvious acute CNS effects. Therefore, 320 mg/kg (-)-borneol was used for inhibition studies.

To determine the optimal dose of TMA that inhibits 2-PPA-CoA formation, rats were pretreated with various doses in corn oil 10 min before 2-PPA administration. Compared to 0.9% saline-pretreatment, 2-PPA-CoA formation was markedly inhibited by 41% by corn oil alone (Figure 5.3), 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. A 500 mg/kg TMA dose in corn oil (1 ml) was found to be optimal, inhibiting 2-PPA-CoA formation by 86% (Figure 5.3), while further increases of the TMA dose did not result in a greater decrease in 2-PPA-CoA formation. Therefore, 500 mg/kg TMA in corn oil was used for inhibition studies.

5.3.3 Inhibitory Studies with (*RS*)-2-PPA

In control rats (corn oil-preteated), formation of 2-PPA-CoA was very rapid and reached an apparent plateau (49 nmol/g liver) by 0.25 h (Figure 5.4A). 2-PPA acyl glucuronidation (sum of (*R*)- and (*S*)-2-PPA-1-*O*-G) in control rats was evident by 0.25 h and maximum concentration (632 nmol/g liver) by 0.5 h (Figure 5.5A). The maximum concentration of 2-PPA acyl glucuronide in livers from (*RS*)-2-PPA-treated rats was nearly 12-fold greater than that of 2-PPA-CoA (Figure 5.5A vs Figure 5.4A), which is consistent with the higher capacity for acyl glucuronidation.

Formation of 2-PPA-CoA thioester in (-)-borneol-treated rats was similar to control rats (corn oil-treated), with 44 nmol/g liver concentration measured after 0.25 h (Figure 5.4B). In contrast, 2-PPA acyl glucuronidation was markedly inhibited by

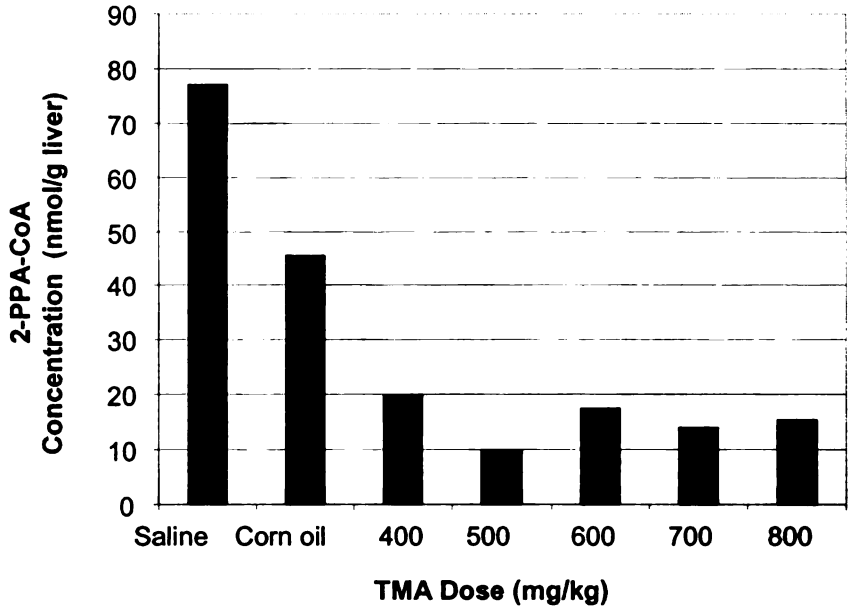


Figure 5.3 Dose-dependent inhibition of 2-PPA-CoA formation by TMA. Rats were pretreated with 0.9% saline (1ml/rat), corn oil (1ml/rat) and various doses of TMA in corn oil (1 ml/rat) 10 min prior to 2-PPA administration (130 mg/kg in 0.9% saline, 0.5 ml/rat). One rat was used for each dosage treatment.

(-)-borneol-treatment (Figure 5.5B). 2-PPA acyl glucuronides were undetectable by HPLC in (-)-borneol-treated rats during the first 1 h. The concentration of 2-PPA acyl glucuronides detected in (-)-borneol-treated rats was 110 ± 109 nmol/g liver at 2 h, significantly lower than in control rats (543 ± 48 nmol/g liver) (Figure 5.5B vs 5.5A). Compared to control rats (corn oil-pretreated), the exposure ($AUC_{(0-2\,h)}$) of 2-PPA acyl

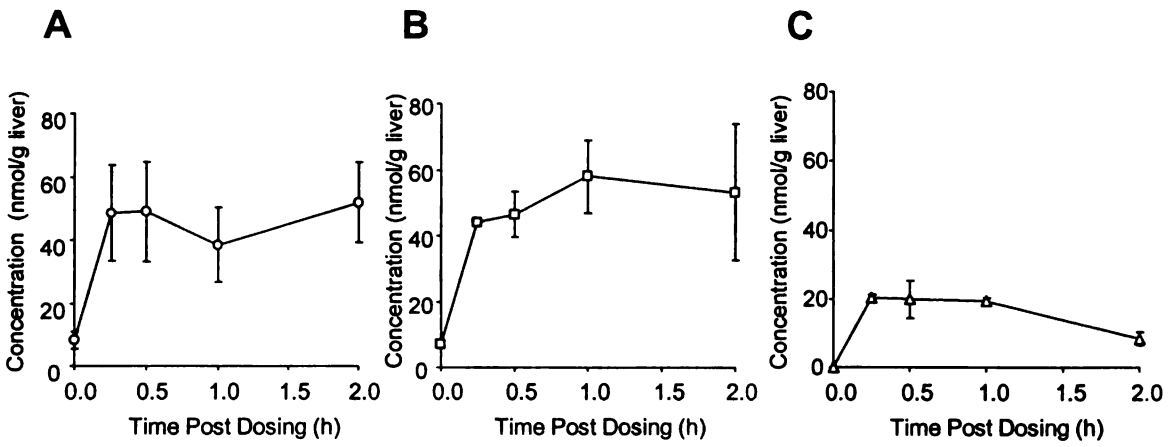
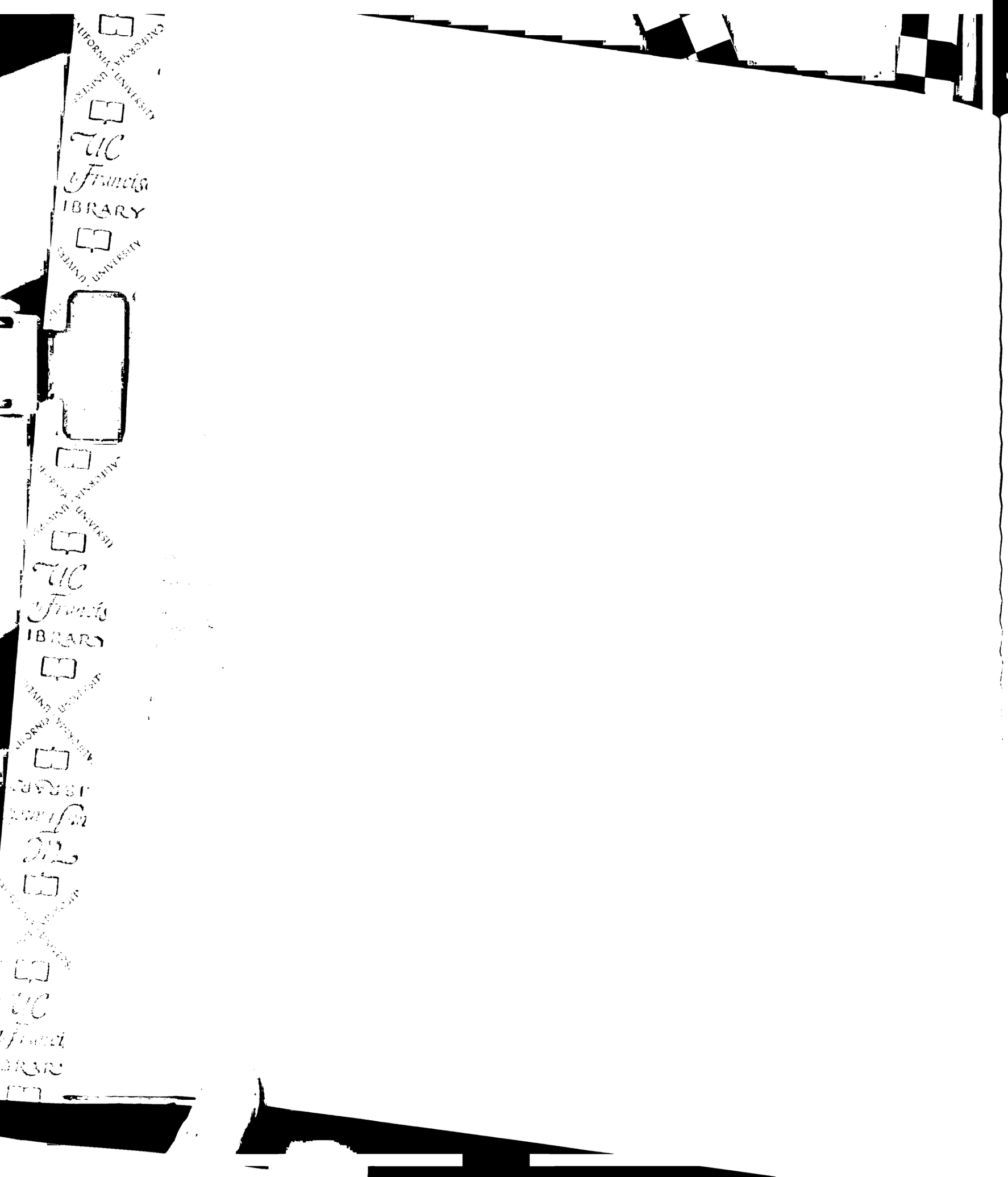


Figure 5.4 Effects of (-)-borneol (B) and TMA (C) on time-dependent 2-PPA-CoA formation in livers from rats dosed with (*RS*)-2-PPA. Rats were pretreated i.p. with A) corn oil (control, 1ml/rat), B) (-)-borneol (320 mg/kg in corn oil, 1 ml/rat) or C) TMA (500 mg/kg in corn oil, 1 ml/rat) 30 min prior to receiving an injection of (*RS*)-2-PPA (130 mg/kg in 0.9% saline, i.p., 0.5 ml/rat). Rats were then killed at the indicated times and their livers collected for homogenization and HPLC analysis. Values are expressed as the mean ± SD (n = 2).

glucuronides to liver proteins over a 2 h period was decreased by (-)-borneol by 95% (Table 5.1). Note that contrary to general belief, acyl glucuronide concentrations can reach similar concentrations to the parent compound when glucuronidation is not inhibited (Figure 5.5A and 5.5C).



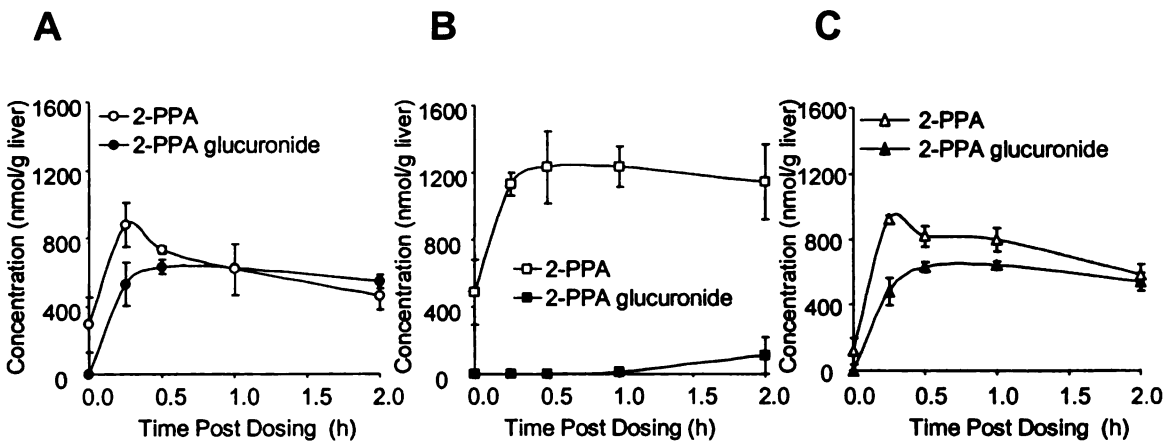


Figure 5.5 Effects of (-)-borneol (B) and TMA (C) on time-dependent 2-PPA acyl glucuronidation in livers from rats dosed with (*RS*)-2-PPA. For experimental conditions see Figure 5.4.

2-PPA-CoA formation was detected in TMA-treated rats, but at much lower concentrations than in controls (Figure 5.4C). TMA-pretreatment decreased 2 h AUC values for 2-PPA-CoA to 36% of controls (Table 5.1). In contrast, formation of 2-PPA acyl glucuronide in (-)-TMA-treated rats was similar to controls (corn oil-treated) (Figure 5.5 and Table 5.1).

Table 5.1. Effects of (-)-borneol and trimethylacetic acid (TMA) on 2-PPA-CoA formation, acyl glucuronidation and irreversible binding to protein in vivo in rat livers.

	2 h post (*RS*)-[1-^{14}C]-2-PPA dosing[a]			AUC $_{(0\text{-}2h)}$ Ratio (treated: control)	
	Irreversible binding (pmol/mg protein)	2-PPA-CoA (nmol/g liver)	2-PPA glucuronide (nmol/g liver)	2-PPA-CoA	2-PPA glucuronide
control	150 ± 5	50.0 ± 8.5	590 ± 34	1.00	1.00
(-)-borneol-treated	115 ± 10[b]	46.8 ± 4.3	196 ± 84[b]	1.15	0.05
TMA-treated	75.8 ± 5.3[b,c]	12.4 ± 1.9[b,c]	491 ± 55[b,c]	0.36	0.99

[a]Rats were treated 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 an i.p. injection of (*RS*)-[1-^{14}C]-2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Livers were collected 2 h post (*RS*)-[1-^{14}C]-2-PPA administration. Values are expressed as the mean ± SD (n = 4).
[b]Significantly different than control using student t-Newman-Keuls method.
[c]Significantly different than (-)-borneol-treated using student t-Newman-Keuls method.

The effects of (-)-borneol and TMA on the metabolic activation of 2-PPA and its irreversible binding to liver proteins were further examined 2 h post (*RS*)-[1-^{14}C]-2-PPA administration. As shown in Table 5.1, the levels of 2-PPA-CoA and acyl glucuronides in livers from rats 2 h after (*RS*)-[1-^{14}C]-2-PPA dosing were consistent with those levels 2 h after cold (*RS*)-2-PPA treatment (Figures 5.5 and 5.6). Time-dependent studies with (*RS*)-[1-^{14}C]-2-PPA were not performed due to the limited availability of (*RS*)-[1-^{14}C]-2-PPA. However, the metabolite concentration-time profiles (AUC) obtained from cold (*RS*)-2-PPA studies appear to be a good estimate of the exposure of reactive metabolites of [1-^{14}C]-2-PPA to liver proteins in (*RS*)-[1-^{14}C]-2-PPA-treated rats.

Pretreatment with (-)-borneol, which almost completely abolished the exposure to 2-PPA acyl glucuronide, decreased irreversible binding by only 23% ($p < 0.05$), whereas,

TMA pretreatment, which decreased exposure of 2-PPA-CoA by 64%, decreased irreversible binding by 49% ($p < 0.05$) (Table 5.1).

5.4 Discussion

Reactive intermediates formed during the metabolism of profen drugs may cause toxicity by binding covalently to proteins (Boelsterli et al., 1995; Pumford and Halmes, 1997). Profen drugs are metabolized by acyl glucuronidation and acyl-CoA formation. Acyl glucuronides are well known to react with proteins in vitro and in vivo (Etter-Kjelsaas and Kuenzle, 1975; Li and Benet, 2002). The reactivity of acyl-CoA thioesters has been less well studied. However, *S*-acyl-CoA thioesters of some carboxylic acids, e.g., nafenopin (Sallustio et al., 2000), clofibric acid (Grillo and Benet, 2002); 2-PPA (Chapter 2 and Li et al., 2002); and naproxen (Olsen et al., 2002) transacylate the sulfhydryl group of GSH and react with proteins, binding the parent acid irreversibly. The present studies were designed to determine the relative contributions of acyl glucuronidation and acyl-CoA formation to the formation of protein adducts of 2-PPA in the liver. 2-PPA was chosen because it is the simplest congener of the profen drugs and is metabolized in rats mainly by glucuronidation and CoA thioester formation.

Selective inhibition of each metabolic pathway led to a decrease in the irreversible binding of 2-PPA to liver proteins (Table 5.1), indicating that both pathways are involved. Binding decreased by 49% ($p < 0.05$) when hepatic exposure to the acyl-CoA thioester was inhibited by 64% with TMA (Table 5.1 and Figure 5.4). In contrast, 95% inhibition of acyl glucuronidation with (-)-borneol decreased irreversible binding by only 23% (Figure 5.5 and Table 5.1). This suggests that the thioester forms more adduct than

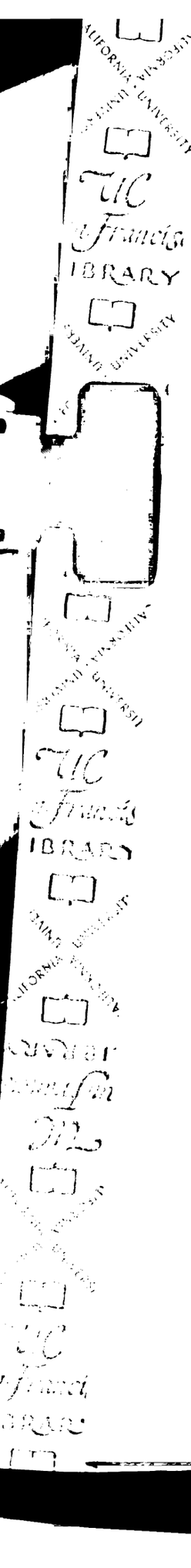
the glucuronide, even though higher concentration of the latter were detected in the liver (Figures 5.5 and 5.6, Table 5.1). This probably reflects the relatively higher chemical reactivity of the thioester, which we have shown to be ~70 fold more reactive towards GSH (a model nucleophile) in vitro in buffer than 2-PPA-1-*O*-G (Chapter 2 and Li et al., 2002).

(-)-Borneol, a monoterpenoid alcohol, has been used as an inhibitor of acyl glucuronidation in hepatocytes in vitro (Porubek et al., 1989; Kretz-Rommel and Boelsterli, 1993) and in rats (Watkins and Klaassen, 1982; Hong et al., 1999). The doses used previously in vivo were 750 – 900 mg/kg (-)-borneol (Watkins and Klaassen, 1982; Hong et al., 1999), which I found to cause CNS effects. Therefore, in preliminary studies I determined the lowest dose of (-)-borneol that would effectively inhibit 2-PPA glucuronidation without evident acute CNS toxicity. This was found to be 320 mg/kg. At this dose, glucuronidation decreased by 95%, yet irreversible binding decreased by only 23%. This suggests that the glucuronide is a contributor, but not the major contributor, to irreversible binding of 2-PPA to hepatic protein in vivo.

Corn oil itself inhibited 2-PPA-CoA formation by 41% (Figure 5.3), but had little effect on 2-PPA acyl glucuronidation (data not shown), indicating the importance of considering the vehicle in drug metabolism study. 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. Since fatty acids are substrates for acyl-CoA formation, these probably inhibit 2-PPA-CoA formation competitively. When rats were dosed with corn oil and TMA together, a further decrease of 2-PPA-CoA formation was observed.

TMA (pivalic acid) is a small branched carboxylic acid, widely used for prodrug production to improve oral bioavailability, e.g., pivampicillin (Binderup et al., 1971) and pivaloyloxymethyl dopa ester (Vickers et al., 1984). Studies on the biological fate of TMA revealed that TMA acyl glucuronide, TMA glycine and carnitine conjugates are the major urinary metabolites in rat urine (Mizojiri et al., 1995). TMA-CoA is believed to be formed in rats since it is the obligatory intermediate for glycine and carnitine conjugates of TMA and rat hepatocytes form TMA-CoA from TMA in vitro (Ruff and Brass, 1991). Consistent with this, I detected TMA-CoA by HPLC in livers from TMA-pretreated rats (Figure 5.1B). Since TMA form both a glucuronide and a CoA thioester, it is interesting that it selectively inhibited formation of 2-PPA-CoA by 64%, but did not inhibit glucuronidation of 2-PPA (Table 5.1).

In conclusion, these studies suggest that CoA thioester formation contributes more to protein adduct formation in the liver than acyl glucuronidation for profen drugs. Whether the concentration and nature of adduct has toxicological significance is not known.



Chapter 6

Effect of Clofibric Acid on the Metabolic Activation of 2-Phenylpropionic Acid in Rats

6.1 Introduction

2-Arylpropionic acids (profen drugs) are a widely used class of nonsteroidal antiinflammatory drugs (NSAIDs), which have a chiral center at the carbon alpha to the carboxyl group. They are dosed therapeutically as racemic mixtures with the notable exception of naproxen. In vitro studies suggested that anti-inflammatory activity resides almost exclusively in the (*S*)-enantiomer (Williams, 1990). However, the two enantiomers of several profen drugs exhibited similar anti-inflammatory properties in vivo (Hutt and Caldwell, 1983), because the inactive (*R*)-enantiomers are unidirectionally inverted in vivo to the active (*S*)-enantiomers (Hutt and Caldwell, 1983; Caldwell et al., 1988). Chiral inversion requires the initial enantioselective formation of acyl-coenzyme A (acyl-CoA) thioester followed by enzymatic epimerization and hydrolysis to generate (*R*)- and (*S*)-enantiomers (Figure 6.1) (Nakamura et al., 1981; Hall and Quan, 1994). Enantioselective activation of the (*R*)-enantiomers to their acyl-CoA thioesters is believed to be the key step, because it accounts for the apparently unidirectional nature of the

process (*R* to *S*, but not *vice versa*) in humans and several animal species (Caldwell et al., 1988).

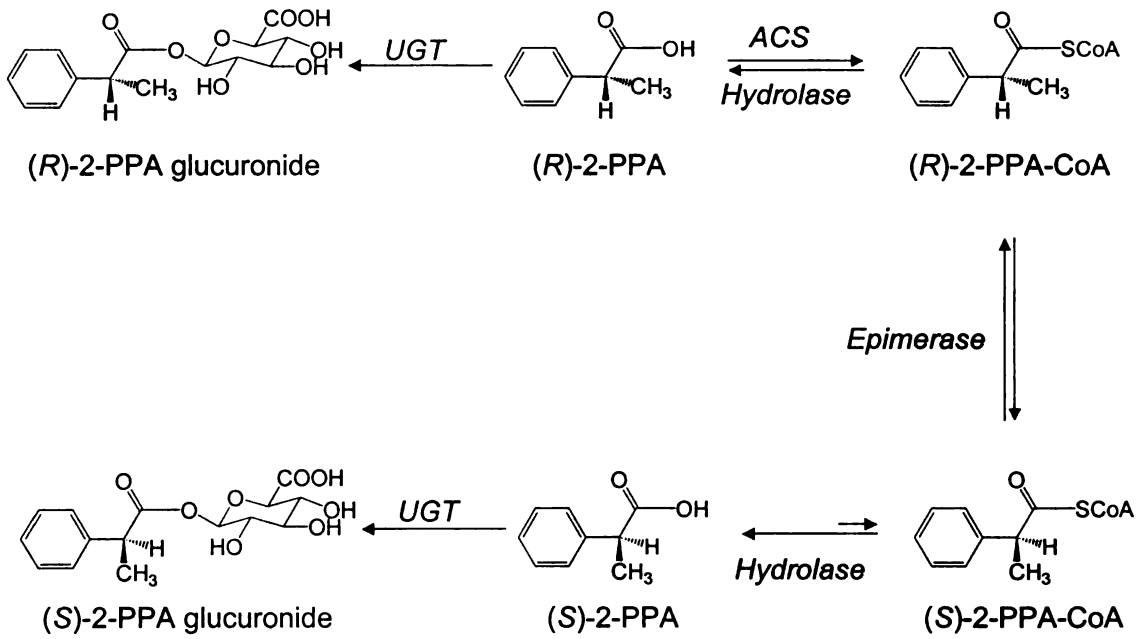


Figure 6.1 Schematic representation of 2-phenylpropionic acid metabolism by inversion, via an acyl-CoA thioester intermediate, and noninversion, i.e., acyl glucuronidation pathway.

The activated acyl-CoA derivatives of profen drugs are intermediates in the formation of amino acid conjugates, acyl carnitine and choline derivatives and hybrid triacylglycerides (Figure 6.2) (Fears, 1985; Hutt and Caldwell, 1990). These processes require a reactive thioester bond, and thus acyl-CoA derivatives are believed to be electrophilic in nature. Studies in our laboratory with 2-phenylpropionic acid (2-PPA, the simplest congener of profen drugs) showed that the acyl-CoA thioester derivative of 2-

PPA is able to acylate the GSH sulfhydryl group non-enzymatically in buffer to form 2-PPA-*S*-acyl glutathione (2-PPA-SG) (Chapter 2 and Li et al., 2002). 2-PPA-*S*-acyl CoA (2-PPA-CoA) was also shown to be reactive towards proteins (such as serum albumin and rat hepatic proteins), forming adducts (Chapter 3).

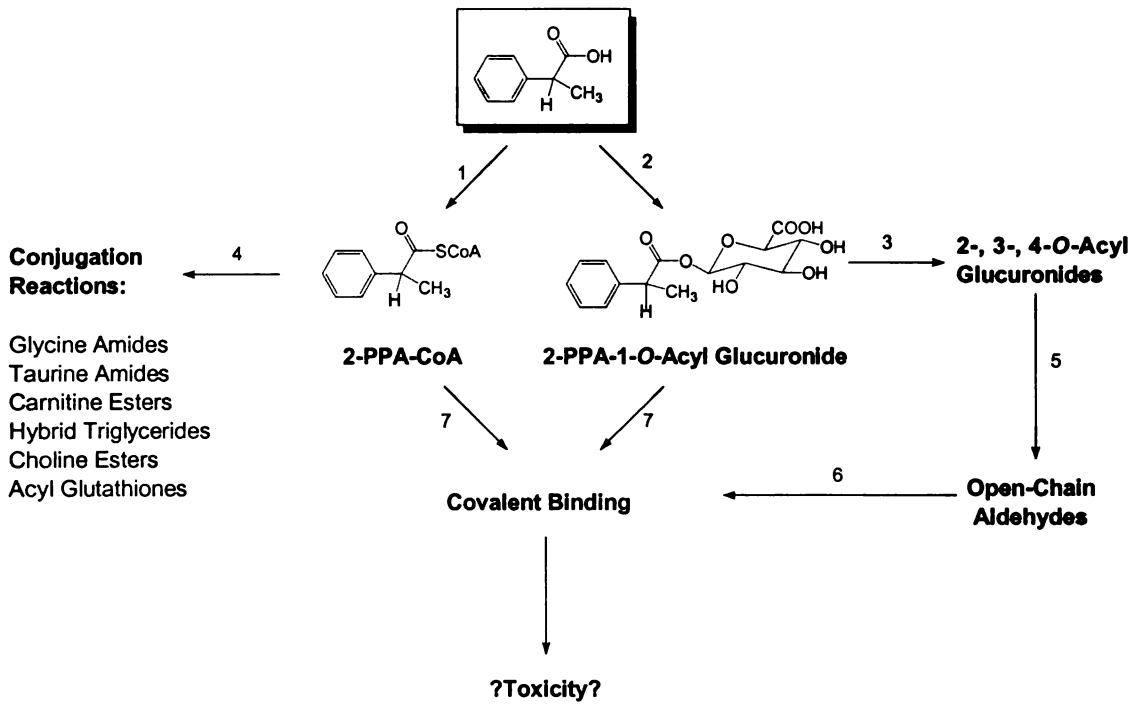


Figure 6.2 Proposed routes in the metabolism of 2-phenylpropionic acids leading to toxic reactive derivatives and non-toxic products. (1) Acyl-CoA formation, (2) acyl glucuronidation, (3) acyl migration, (4) conjugation reactions, (5) ring-chain tautomerism, (6) Schiff-base formation with proteins and (7) transesterification.

Most profens are metabolized to acyl glucuronides and acyl-CoA thioesters, which are reactive and believed to form covalent adduct with proteins in vivo (Figure 6.2). We characterized and compared these two metabolic activation pathways for 2-PPA in freshly isolated rat hepatocytes (Chapter 4) and in vivo in rats (Chapter 5). Enantioselective studies with (*R*)- and (*S*)-2-PPA in hepatocytes showed that covalent binding to protein was enantioselective for the (*R*)-isomer, which correlated well with the enantioselectivity of acyl-CoA formation, but not with acyl glucuronidation (Chapter 4). Inhibition studies with 2-PPA in hepatocytes (Chapter 4) and in vivo in rats (Chapter 5) showed that inhibition of acyl-CoA formation by trimethylacetic acid led to a greater decrease in covalent binding to hepatic protein than inhibition of acyl glucuronidation by (-)-borneol. Together, all these findings suggest that metabolic activation of 2-PPA by acyl-CoA formation contributes more to protein adduct formation in the liver than acyl glucuronidation.

Due to the role of acyl-CoA thioester, we hypothesized that hepatic adduct formation might be sensitive to agents that influence its formation. Clofibric acid (CA), a hypolipidemic agent, is known to be a potent peroxisomal proliferator in rats (Hawkins et al., 1987). CA induces several hepatic enzymes associated with fatty acid metabolism, including acyl-CoA synthetases, the enzymes that catalyze the formation of acyl-CoA thioesters (Alegret et al., 1994; Schoonjans et al., 1993). A significant increase of free CoA, a cofactor that is essential for acyl-CoA formation, was observed in the livers of rats treated with CA (Horie et al., 1986). Shirley et al. (1994) also reported that formation of the acyl-CoA thioester of ibuprofen was markedly increased in freshly isolated hepatocytes from CA-treated rats compared to controls. Therefore, it is plausible that CA

treatment could increase covalent binding of 2-PPA by increasing the exposure of liver proteins to 2-PPA-CoA thioester. The present studies were designed to test this.

6.2 Experimental Procedures

6.2.1 Chemicals

(*RS*)-2-PPA, (*R*)-2-PPA, perchloric acid (70%), EDTA, clofibric acid (CA, 2-[*p*-chlorophenoxy]isobutyrate) were purchased from Aldrich Chemical Co. (Milwaukee, WI). $NaHCO_3$ and diethyl ether from Fisher Scientific (Fair Lawn, NJ). CoA, ATP, $MgCl_2$, dithiothreitol (DTT), Triton X-100, Tris-HCl and trifluoroacetic acid (TFA) were obtained from Sigma Co. (St. Louis, MO). Hionic-Fluor scintillation fluid was purchased from Packard BioScience Co. (Meriden, CT). (*RS*)-[1-^{14}C]-2-PPA and [1-^{14}C]-palmitic acid were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Synthetic 2-PPA-CoA and the biosynthetic 2-PPA-1-*O*-G were available from previous studies in this laboratory (Chapter 2 and Li et al., 2002). All solvents used for HPLC analysis were of chromatographic grade.

6.2.2 Animals

Male Sprague-Dawley rats (200 ~250 g) were purchased from B & K Universal (Livermore, CA), maintained in a controlled housing environment with 12-h light/dark cycles, and received standard laboratory chow and water ad libitum. Rats were allowed at least 3 days to become acclimated to the housing conditions before use in experiments.

All animal studies were approved by the University of California San Francisco Committee on Animal Research.

6.2.3 Enzyme Activities in Rat Liver Homogenate

Rats (n = 5) were dosed i.p. with either clofibric acid (160 mg/kg in 0.1 M sodium bicarbonate, pH 7.8, 1ml/rat) or vehicle (0.1 M sodium bicarbonate, pH 7.8, 1 ml/rat) for 7 days. On Day 8, the rats were anesthetized with ether, the abdominal cavities opened and the livers perfused with ice-cold saline. Perfused livers were rapidly removed, weighed and frozen immediately in liquid nitrogen. Rat liver homogenate was prepared as described previously (Chapter 3). To determine (*R*)-2-PPA-CoA synthetase activity, (*R*)-2-PPA (1 mM) was incubated with 0.25 mg liver homogenate protein, 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM $MgCl_2$ and 2.5 mM ATP in a final volume of 0.5 mL (Chapter 3). After a 3 min preincubation, reactions were initiated by addition of ATP, allowed to proceed for 30 min at 37°C, then stopped by addition of 50 μL perchloric acid (7%). Under the above conditions, the formation of 2-PPA-CoA was linear with incubation time and protein concentration, while enzymes were saturated with cofactors CoA and ATP. After centrifugation at 10,000 *g* for 5 min, the supernatants were neutralized with 1M NaOH and analyzed by reverse-phase HPLC.

Palmitoyl-CoA synthetase activity in rat liver homogenate was determined with [1-^{14}C]-palmitic acid by the previously described method (Krisans et al., 1980) with a minor modification. Briefly, incubations of [1-^{14}C]-palmitic acid (100 μM, 5.2 Ci/mol) with rat liver homogenate (5 μg) 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 $MgCl_2$, and 2.5 mM ATP in a final volume of 0.2 ml. 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. The incubation was terminated after 5 min and [1-^{14}C]-palmitoyl-CoA was quantified as previously described (Krisans et al., 1980).

6.2.4 Metabolic Activation Studies with 2-PPA

One group of rats (n = 13) was dosed i.p. with clofibric acid (160 mg/kg in 0.1 M sodium bicarbonate, pH 7.8, 1ml/rat) for 7 days. The control group (n = 13) was treated only with vehicle (0.1 M sodium bicarbonate, pH 7.8, 1 ml/rat). On day 8, the rats were given a single i.p. dose of (*RS*)-2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). After administration of 2-PPA, two rats were decapitated at times 0, 0.25, 0.5, 1 and 2 h for both CA and control pretreatment groups. An additional 6 rats, 3 each for CA and control pretreatments, received (*RS*)-[1-^{14}C]-2-PPA (0.1 Ci/mol, 130 mg/kg in 0.9% saline, 0.5 ml/rat) and were killed 2 h later. Their tissues, including liver, kidney, lung, heart, brain, spleen and skeletal muscle were removed, rinsed of blood, and immediately frozen in liquid nitrogen. Tissues were stored at -80°C for the analysis of covalent binding. 2-PPA-CoA formation and acyl glucuronidation in rat livers were quantified by reverse phase HPLC.

6.2.5 Determination of 2-PPA, 2-PPA-CoA and 2-PPA Acyl Glucuronide in Rat Livers

2-PPA-CoA formation and acyl glucuronidation in rat livers were determined as described previously (Chapter 5). Briefly, frozen rat liver (1.0 g) was homogenized in 1.5 ml of potassium phosphate buffer (0.05 M, pH 5). The resultant liver homogenate was immediately denatured by 0.75 ml $HClO_4$ (7%), mixed vigorously and centrifuged (10,000 *g*, 10 min). The supernatant was neutralized with 1M NaOH and analyzed by reverse-phase HPLC for the formation of 2-PPA-CoA thioester. For the analysis of 2-PPA acyl glucuronides and 2-PPA in rat livers, frozen rat liver (0.5 g) was homogenized in 0.5 ml of potassium phosphate buffer (0.05 M, pH 5). The resultant liver homogenate was immediately denatured by 0.5 ml acetonitrile. After centrifugation at 10,000 *g* for 10 min, the supernatant was analyzed by HPLC for determination of 2-PPA and 2-PPA acyl glucuronides.

6.2.6 HPLC Analysis

HPLC analysis was carried out on a Shimadazu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadazu Corp., Japan) with a Shimadazu SCL-10A controller and a Shimadazu SPD-10A UV-Vis detector. The formation of 2-PPA-CoA in rat liver and liver homogenate was analyzed on a SB-C_8 Zorbax column (150 x 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. 2-PPA acyl glucuronidation in livers of 2-PPA-treated rats was quantified by reverse phase HPLC

using an isocratic elution with 0.1% trifluoroacetic acid (TFA) and 15% acetonitrile on a Microsorb-MV C_{18} column (150 x 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), which gave a good separation of (*R*)-2-PPA acyl glucuronide from the (*S*)-diastereoisomer. Separation of (*R*)- and (*S*)-2-PPA was achieved by reverse-phase HPLC using 0.1% TFA in 30% acetonitrile on a chiral column (Chiralcel OJ-R, 4.6 x 150 mm, Chiral Technologies Inc., Exton, PA) at a flow rate of 0.8 ml/min and with UV detection at 226 nm. Quantitative measurements of 2-PPA-CoA, 2-PPA-acyl glucuronides and 2-PPA were made using a standard curve by adding synthetic 2-PPA-CoA, biosynthetic 2-PPA-1-*O*-G or 2-PPA standards to liver homogenates from untreated rats.

6.2.7 Covalent Binding of (*RS*)-[1-^{14}C]-2-PPA to Tissue Proteins

Covalent binding of 2-PPA to proteins was measured by scintillation counting of exhaustively washed protein precipitates as described previously (Chapter 3) with minor modifications. Briefly, frozen tissue (1.0 g) was homogenized in 1.5 ml of potassium phosphate buffer (0.05 M, pH 5). The homogenate was immediately denatured with 0.75 ml $HClO_4$ (7%), mixed vigorously and centrifuged (1500 *g*, 10 min). Protein pellets were washed 7 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 (1500 *g*, 5 min) until there no detectable radioactivity in the supernatant. Washing continued with methanol/ethyl ether (3:1, v/v, 7 x 6 ml), followed by 80% aqueous methanol (7 x 6 ml). No radioactivity was detected in the supernatants of the last wash. After the final supernatants were removed, the pellets were left to dry at room temperature for 2 h and were then heated in 1M NaOH (1.5 ml) at 80°C overnight. The clear solution (1.25 ml)

was counted in 10 ml of Hionic-Fluor scintillation fluid. Protein concentrations were determined using the BCA protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as standard, following the manufacturer's instructions. Covalent binding is expressed as pmol of bound 2-PPA per mg of protein.

6.3 Results

6.3.1 Effect of CA on Body Weight, Liver Weight and Acyl-CoA Synthetase Activities

To access the effect of CA treatment on the acyl-CoA synthetase activities, rats were treated i.p. with 160 mg/kg of CA, a dose equivalent to that used previously (Alegret et al., 1994). Palmitoyl-CoA synthetase activity increased during 7 days, but not on further treatment (Alegret et al., 1994). Therefore, 7-day treatment was used in the present study. Body weight increased steadily during the 7-day treatment, but there was no significant difference between CA-treated and control rats (Table 6.1). In CA-treated rats liver weight increased 22% more than in control rats ($p < 0.02$, Table 6.1), because of hepatomegaly (Hawkins et al., 1987). Rat liver homogenate isolated from CA-treated rats had 76% higher palmitoyl-CoA synthetase activity ($p < 0.001$) than controls, similar to that previously reported (Alegret et al., 1994). Formation of 2-PPA-CoA from (*R*)-2-PPA by liver homogenate was markedly (6.9 fold) enhanced by CA-treatment ($p < 0.001$) under saturating conditions with respect to CoA and ATP (Table 6.1).

Table 6.1 Effect of clofibric acid on body weight, liver weight, palmitoyl-CoA synthetase activity and (*R*)-2-PPA-CoA synthetase activity in liver homogenate in the rat.

	Control Rats	CA-Treated Rats
% increase in body weight[a]	23 ± 4	20 ± 3
liver weight (g)	11.7 ± 0.3	14.3 ± 1.3[b]
palmitoyl-CoA synthetase activity (nmol/min/mg)	39.8 ± 2.6	69.9 ± 7.9[c]
(*R*)-2-PPA-CoA synthetase activity (pmol/min/mg)	45.2 ± 13.7	357 ± 50[c]

Data are expressed as means ± SD (n = 5)
[a]Body weight was measured immediately before experiments.
[b]$p < 0.02$ compared to corresponding control group, using t test
[c]$p < 0.001$compared to corresponding control group, using t test

6.3.2 Effect of CA on 2-PPA Acyl-CoA Formation, Acyl Glucuronidation and Covalent Binding In Vivo in Rat Livers

Consistent with the increased 2-PPA-CoA synthetase activity in rat liver homogenate, CA-treated rats had a significantly higher levels of hepatic 2-PPA-CoA thioester than controls (Figure 6.3A). The exposure of liver proteins to 2-PPA-CoA over a 2 h period was markedly increased by CA ($p < 0.01$) (Figure 6.3A and Table 6.2). Conversely, treatment with CA for 7 days slightly decreased the exposure of 2-PPA acyl glucuronides to rat liver (Figure 6.3B and Table 6.2). These changes led to a 25% increase in covalent binding of 2-PPA to hepatic proteins ($p < 0.05$) in rats pretreated with CA 2 h after 2-PPA administration (Figure 6.3C).

6.3.3 Covalent Binding of 2-PPA to Rat Extrahepatic Tissues

Having demonstrated that CA increases covalent binding of 2-PPA to rat liver

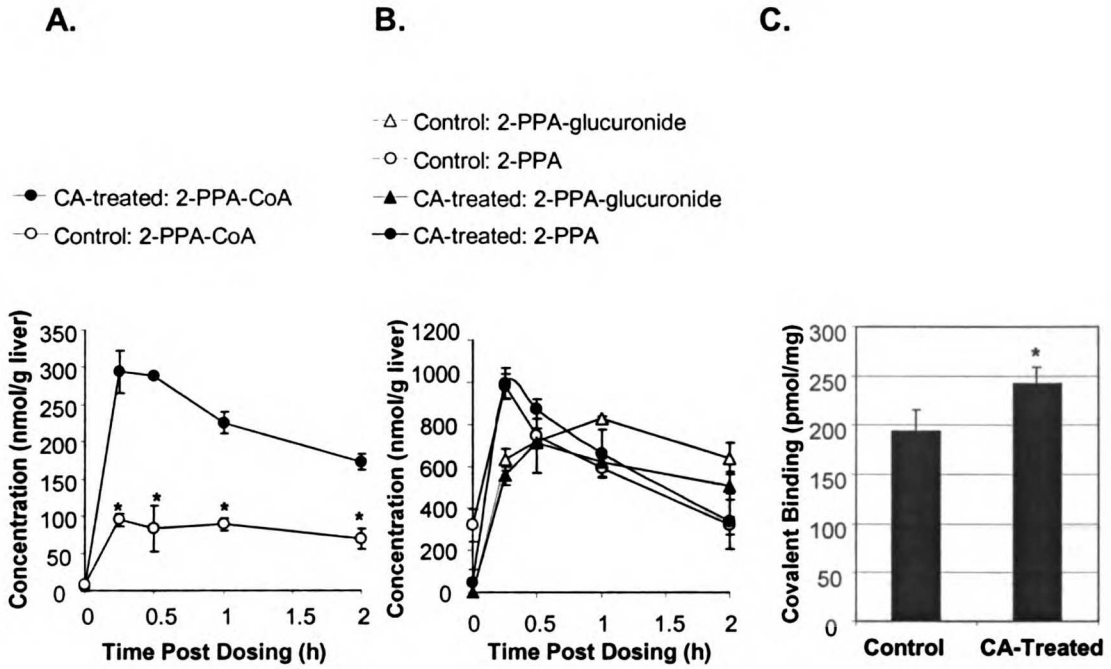


Figure 6.3 Effects of clofibric acid (CA) on 2-PPA-CoA formation (A), acyl glucuronidation (B) and irreversible binding of 2-PPA to liver proteins (C) in vivo in rats. Rats were pretreated i.p. with CA (160 mg/kg) or vehicle (control) for 7 days. On Day 8, rats received an i.p. injection of 2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Livers were collected at the indicated times after 2-PPA administration. Values are expressed as the mean ± SD ($n \geq 2$). * $p < 0.05$

proteins, we examined other tissues. Figure 6.4 shows covalent binding of 2-PPA in various tissues 2 h after [1-^{14}C]-2-PPA administration. Radioactivity was detectable in exhaustively washed protein precipitates from liver, kidney, lung, heart, brain, spleen and skeletal muscle (Figure 6.4). In control rats (vehicle-treated), irreversible binding to protein was highest in liver and kidney, and lowest in skeletal muscle. CA selectively

increased adduct formation in rat liver by 25% ($p < 0.05$), but had no significant effect in other rat tissues. Irreversible binding in skeletal muscle appeared to be increased by CA, but the increase was not statistically significant (Figure 6.4).

Table 6.2 Effects of CA on 2-PPA-CoA formation, acyl glucuronidation and covalent binding *in vivo* in rat livers[a]

	$AUC_{(0\text{-}2h)}$ (nmol·h/g liver)			Covalent binding 2h after injection (pmol/mg protein)
	2-PPA-CoA	2-PPA glucuronide	2-PPA	
CA-treated	440 ± 22[b]	1120 ± 30	1300 ± 100	243 ± 21[c]
Control	153 ± 16	1400 ± 70	1180 ± 40	195 ± 16
CA/C Ratio	2.88	0.79	1.10	1.25

[a]Rats were pretreated i.p. with CA (160 mg/kg) or vehicle (control) for 7 days. On Day 8, rats received an i.p. injection of 2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Following 2-PPA administration, livers were collected at the indicated times in Figure 3A and B. Values are expressed as the mean ± SD (n ≥ 2).
[b]$p < 0.01$.
[c]$p < 0.05$.

6.3.4 Effect of CA on Enantiomeric Composition of 2-PPA and 2-PPA Acyl Glucuronides in Rat Liver

Like other widely used profen drugs, such as ibuprofen and fenoprofen (Caldwell et al., 1988), the (*R*)-enantiomer of 2-PPA undergoes unidirectional chiral inversion to form the (*S*)-isomer (Yamaguchi and Nakamura, 1985). Enantioselective formation of (*R*)-2-PPA-CoA is believed to be the key step mediating this chiral inversion (Nakamura et al., 1981). Since CA markedly increased 2-PPA-CoA formation in rat liver, the effect of CA on the enantiomeric composition of 2-PPA and the glucuronides was examined. Figure 6.5 shows the concentration-time curves for (*R*)- and (*S*)-2-PPA acid and glucuronides in liver following i.p. administration of (*RS*)-2-PPA. In control rats,

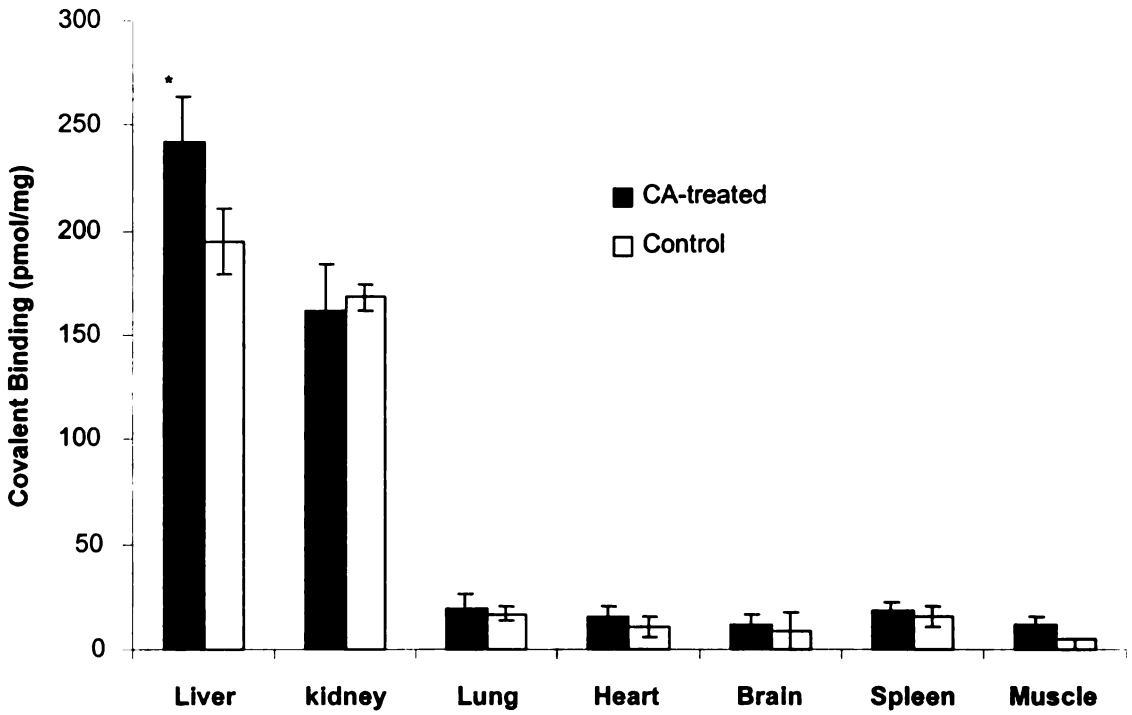


Figure 6.4 Covalent binding of (*RS*)-[1-^{14}C]-2-PPA to tissue proteins in vivo in clofibric acid (CA)-treated and control rats. Rats were pretreated i.p. with CA (160 mg/kg) or vehicle (control) for 7 days. On Day 8, rats received an i.p. injection of (*RS*)-[1-^{14}C]-2-PPA (0.1 Ci/mol, 130 mg/kg) and were sacrificed 2 h later. Values are expressed as the mean ± SD (n = 3). * $p < 0.05$.

concentrations of both (*S*)-2-PPA acid and its glucuronide in the liver were significantly greater than those of the corresponding isomers. The *R/S* ratio of total 2-PPA (2-PPA plus glucuronide) decreased gradually with time from 1.0 at time 0 h to 0.517 2 h after (RS)-2-PPA administration. CA treatment further decreased the *R/S* ratios by selectively increasing the level of (*S*)-2-PPA and decreasing (*R*)-2-PPA-1-*O*-G. The amounts of (*R*)-2-PPA and (*S*)-2-PPPA-1-*O*-G in the liver were not significantly changed by CA. As

indicated in Figure 6.5C, the *R/S* ratio significantly decreased from 0.72 in control rats to 0.38 in CA-treated in 2 h (Figure 6.5).

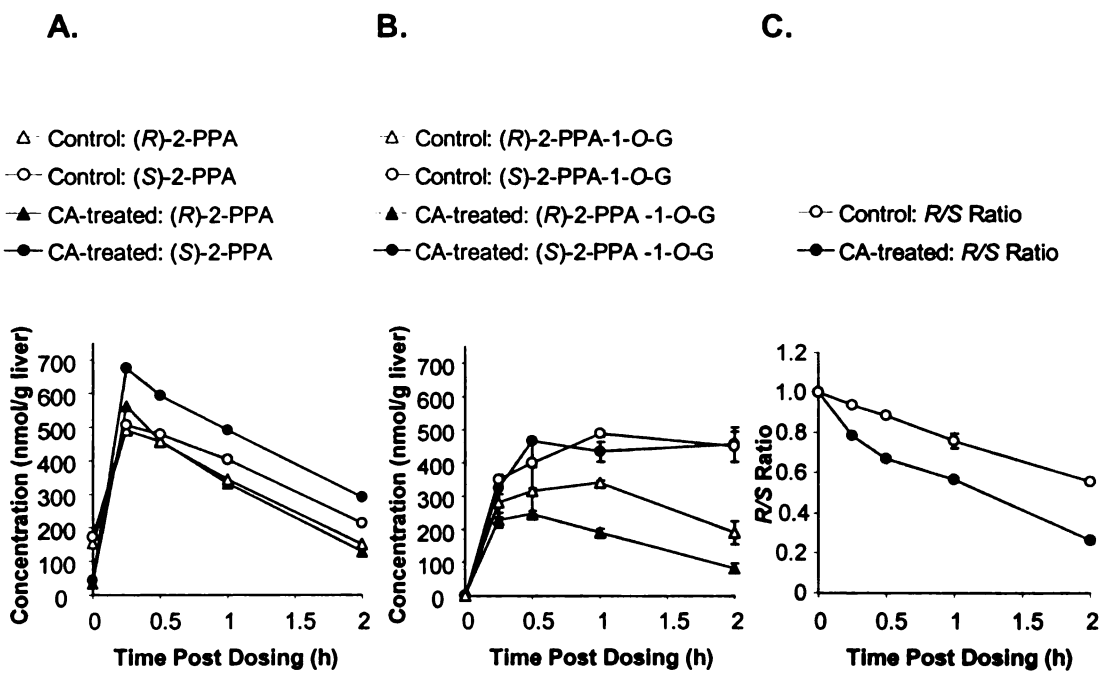


Figure 6.5 Effect of CA on enantiomeric composition of 2-PPA (A), 2-PPA acyl glucuronides (B), and the *R/S* ratio of total 2-PPA (2-PPA acid plus acyl glucuronide) (C) in rat liver. Rats were pretreated i.p. with CA (160 mg/kg) or vehicle (control) for 7 days. On Day 8, rats received an i.p. injection of 2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Following 2-PPA administration, livers were collected at the indicated times. Values are expressed as the mean ± SD ($n \geq 2$).

indicated in Figure 6.5C, the *R/S* ratio significantly decreased from 0.72 in control rats to 0.38 in CA-treated in 2 h (Figure 6.5).

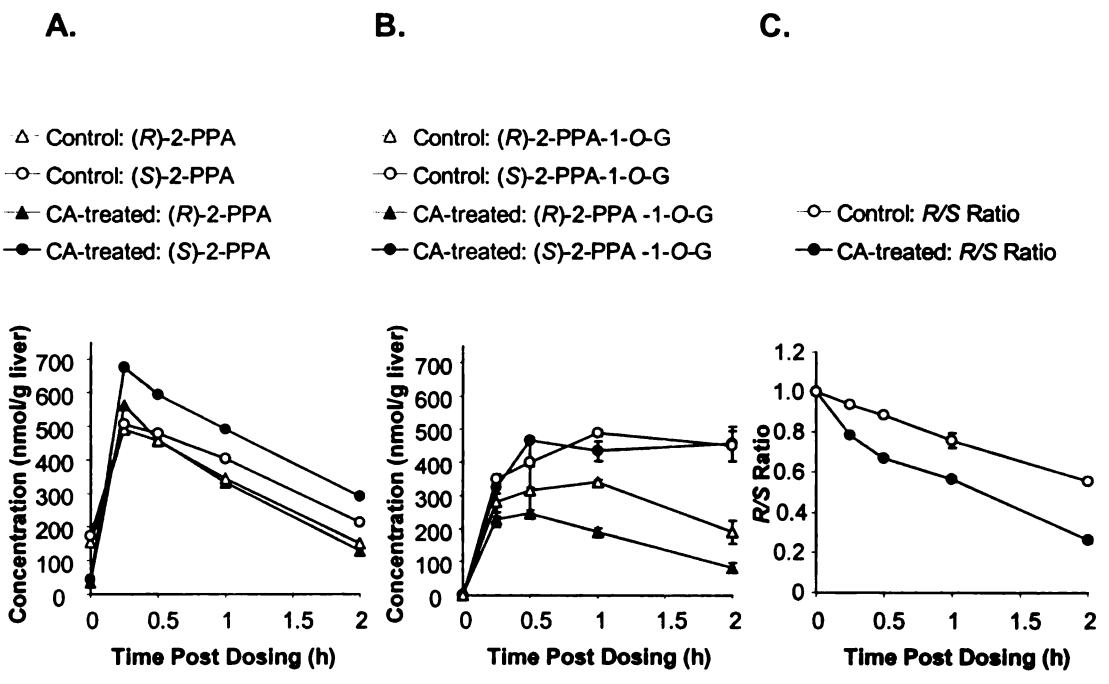


Figure 6.5 Effect of CA on enantiomeric composition of 2-PPA (A), 2-PPA acyl glucuronides (B), and the *R/S* ratio of total 2-PPA (2-PPA acid plus acyl glucuronide) (C) in rat liver. Rats were pretreated i.p. with CA (160 mg/kg) or vehicle (control) for 7 days. On Day 8, rats received an i.p. injection of 2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat). Following 2-PPA administration, livers were collected at the indicated times. Values are expressed as the mean ± SD ($n \geq 2$).

6.4 Discussion

The present studies were designed to examine whether CA treatment can increase the irreversible binding of the model profen, 2-PPA, to proteins in vivo. CA is known to induce peroxisomal proliferation in rats. The typical manifestations of CA induction are hepatomegaly (Hawkins et al., 1987) and increases in expression and activity of the enzymes involved in the metabolism of fatty acids, including acyl-CoA synthetase (Alegret et al., 1994; Schoonjans et al., 1993). We observed significant increases in both liver weight and palmitoyl-CoA synthetase activity with CA treatment. As shown in Table 6.1, the increase in liver weight was 22% greater in CA-treated rats than controls ($p < 0.02$). Palmitoyl-CoA synthetase activity in liver homogenate was significantly increased by 76% ($p < 0.001$) by CA-treatment. Compared to the induction of palmitoyl-CoA synthetase, administration of CA for 7 days caused a much greater increase of the ability of rat liver homogenate to catalyze the formation of 2-PPA-CoA from (*R*)-2-PPA (7.9 fold, $p < 0.001$), suggesting that acyl-CoA synthetases other than palmitoyl-CoA synthetase may be more susceptible to CA induction. This is consistent with our previous kinetic studies in rat liver homogenate, which indicated that more than one acyl-CoA synthetase is involved in 2-PPA-CoA formation (Chapter 3).

In addition to the increased "2-PPA-CoA" synthetase activity in liver homogenate, CA-treated rats had significantly higher levels of hepatic 2-PPA-CoA thioester relative to controls. (Figure 6.3A). The exposure of liver proteins to 2-PPA-CoA over a 2-h period was 2.88 greater in treated animals than controls (Table 6.2). This, however, was less than the 7.9 fold increase in "2-PPA-CoA" synthetase activity observed in vitro in rat liver homogenate (Table 6.1). As indicated in Figure 6.1, acyl-

CoA thioester can be hydrolyzed back to the acid by acyl-CoA hydrolase. The activated acyl-CoA thioester is also used for formation of amino acid conjugates, acyl carnitine and choline derivatives (Figure 6.2). Therefore, the level of 2-PPA-CoA thioester in rat liver depends on not only the formation of 2-PPA-CoA thioester, but also on its subsequent metabolism. CA induces acyl-CoA hydrolase (Berge and Bakke, 1981; Alegret et al., 1994) and carnitine acyl transferase, the enzyme that catalyzes the formation of acyl-carnitine from acyl-CoA thioester (Tosh et al., 1989), in addition to its effect on acyl-CoA synthetase. Consequently, the moderate increase in 2-PPA-CoA exposure in vivo in the liver may reflect differential contribution of CA to these competitive pathways. This hypothesis is currently under investigation in our laboratory.

In addition to induction of the enzymes involved in the metabolism of fatty acids, CA also specifically induces an isoform of UDP-glucuronosyl transferase (UGT), UGT 1A1, that catalyzes the formation of bilirubin and other glucuronides (Ritter, 2000). However, CA has little inductive effect on other UGTs, including those that catalyze acyl glucuronidation of NSAIDs (Fournel-Gigleux et al., 1988; Ritter, 2000). It has also been shown that CA treatment slightly decreases 2-PPA acyl glucuronidation in rat liver microsomes (Fournel-Gigleux et al., 1988). Consistent with these in vitro results, we observed a 21% reduction in the exposure of 2-PPA acyl glucuronides to liver proteins in vivo with CA pretreatment (Figure 6.3B).

Irreversible binding of 2-PPA to liver protein was significantly increased 25% by CA ($p < 0.05$) (Figure 6.3C). From this finding, it is impossible to determine whether 2-PPA-CoA or 2-PPA acyl glucuronide was chiefly responsible for covalent binding. That would require concomitant binding measurements in the presence of inhibitors of each

pathway under CA induced conditions, as we described in Chapters 4 and 5. However, we have demonstrated that CA treatment does increase irreversible binding by increasing the exposure of 2-PPA-CoA thioester to liver proteins. Therefore CA may increase the risk of the idiosyncratic toxicity associated with the use of profen drugs.

Covalent binding of [1-^{14}C]-2-PPA was also detected in organs other than liver, including kidney, lung, heart, brain, spleen and skeletal muscle. The greatest extent of covalent binding was found in the liver and kidney, the major sites of chiral inversion of 2-PPA and thus of 2-PPA-CoA biosynthesis (Yamaguchi and Nakamura, 1987). However the effect of CA treatment was mainly in the liver. This is consistent with the observation by Schoonjans et al. (1993) that liver is the most responsive tissue to fibrate.

In CA-treated rats, there was a significant increase in hepatocellular exposure to 2-PPA-CoA thioester and consequently a significant increase in metabolic chiral inversion of 2-PPA (Figures 6.3 and 6.5). CA-treatment markedly increased the fractional inversion of (*R*)-2-PPA to (*S*)-enantiomer in the liver, by selectively increasing the level of (*S*)-2-PPA and decreasing (*R*)-2-PPA-1-*O*-G (Figure 6.5).

Collectively, the present studies provide evidence for a potential clinical interaction between clofibric acid and profen drugs. Enhanced exposure to (*S*)-profen due to an increased inversion of (*R*)-isomer could be clinically important and contribute to both the desired therapeutic effects and their toxicity profile (e.g. gastrointestinal ulceration). More importantly, the present studies demonstrate that CA treatment can increase irreversible binding to hepatic proteins and possibly the risk of idiosyncratic toxicity.

Chapter 7

Effects of Diabetes on the Metabolic Activation of 2-Phenylpropionic Acid in Rats

7.1 Introduction

Many 2-arylpropionic acids are metabolized to acyl glucuronides (Li and Benet, 2002) and acyl-CoA thioesters, which are both reactive and believed to form covalent adducts with proteins *in vivo* (Chapters 2-5; Smith et al., 1986; Sallustio et al., 2000; Li and Benet, 2002). We hypothesize that hepatic adduct formation might be influenced by factors that influence these two metabolic activation pathways. Experimental models of diabetes mellitus are characterized by a number of features that may lead to changes in metabolic activation of 2-arylpropionic acids. For example in streptozotocin-treated rats, a model of insulin-dependent diabetes mellitus (type 1), there are marked increases in both cellular CoASH concentration (Horie et al., 1986) and the activity of long-chain acyl-CoA synthetase, the enzyme responsible for the catalysis of acyl-CoA thioester (Asayama et al., 1999). Diabetic rats also show altered glucuronidation of many drugs (Eacho et al., 1981; Price and Jollow, 1982; Grant and Duthie, 1987). The present studies were designed to examine the effect of streptozotocin-induced diabetes on the metabolic

activation of a model compound, 2-phenylpropionic acid (2-PPA), in hepatocytes to explore the factors that may influence 2-PPA adduct formation.

7.2 Experimental Section

7.2.1 Chemicals

(*RS*)-2-PPA, (*R*)-2-PPA and perchloric acid (70%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium citrate, $NaHCO_3$ and diethyl ether from Fisher Scientific (Fair Lawn, NJ). Streptozotocin and trifluoroacetic acid (TFA) were obtained from Sigma Co. (St. Louis, MO). Hionic-Fluor scintillation fluid was purchased from Packard BioScience Co. (Meriden, CT). (*RS*)-[1-^{14}C]-2-PPA was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Synthetic 2-PPA-CoA and the biosynthetic 2-PPA-1-*O*-G were available from previous studies in this laboratory (Chapter 2 and Li et al., 2002). Blood glucose was measured using the One Touch® monitoring kit from Lifescan (Milpitas, CA). All solvents used for HPLC analysis were of chromatographic grade.

7.2.2 Animal Treatment

Male Sprague-Dawley rats (200 ~250 g) were purchased from B & K Universal (Livermore, CA), maintained in a controlled housing environment with 12-h light/dark cycles, and received standard laboratory chow and water ad libitum. Rats were allowed at least 3 days to become acclimated to the housing conditions before use in experiments.

All animal studies were approved by the University of California San Francisco Committee on Animal Research. Type I diabetes was induced in the rats by a single i.p. injection of streptozotocin (65 mg/kg) freshly dissolved in 50 mM ice cold sodium citrate buffer, pH 4.5 (n = 4). Control rats were given an injection of buffer only (n = 4). Body weight and blood glucose were monitored prior to induction of diabetes and weekly throughout the study period. On day 21 following streptozotocin administration, rats were utilized for experiments.

7.2.3 In Vitro Studies with Rat Hepatocytes

Freshly-isolated rat hepatocytes were prepared according to published procedures (Moldeus et al., 1978). Greater than 85% viability was achieved, as assessed by trypan blue exclusion. Incubations of hepatocytes (4 million cells/ml) with (*RS*)-[1-^{14}C]-2-PPA (0.2 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 were taken at 2, 10, 20, 30, 60, 120 and 180 min and analyzed for acyl-CoA formation and acyl glucuronidation.

After the addition of 2-PPA free acid to hepatocyte incubations, aliquots (0.5 mL) were taken at indicated times and quenched with perchloric acid (7%, 0.25 mL) for determination of acyl-CoA formation. The mixtures were centrifuged (10,000 *g*, 10 min) and the supernatants neutralized with 1 M NaOH and analyzed by reverse-phase HPLC, as described in Chapter 4. For the analysis of 2-PPA acyl glucuronidation in hepatocytes, 0.5 mL aliquots from incubation mixture were taken at indicated time points and quenched with acetonitrile (0.5 mL) and HCl (1M, 12.5 μL). After centrifugation

(10,000 *g*, 10 min), supernatants were analyzed by reverse-phase HPLC, as described previously (Chapter 4).

The effects of diabetes on covalent binding were assessed after 3 h incubation. Aliquots (4 mL) were taken at 3 h and quenched with perchloric acid (7%, 2 mL). Covalent binding of 2-PPA to protein was measured by scintillation counting of exhaustively washed hepatocyte protein precipitates, as described in Chapter 4.

7.3 Results

7.3.1 Effect of Diabetes on 2-PPA Acyl-CoA Formation, Acyl Glucuronidation and Covalent Binding in Freshly Isolated Hepatocytes

All rats treated with streptozotocin had lower body weights and higher blood glucose levels than controls. Three weeks following streptozotocin treatment blood glucose levels were 138 ± 14 mg/dl in control animals and 389 ± 44 mg/dl in the diabetic animals ($p < 0.005$). The body weights of diabetic animals were 284 ± 19 g, which was significantly lower than controls (356 ± 9 g, $p < 0.005$).

Diabetic rats showed a significant induction of 2-PPA-CoA thioester formation in rat hepatocytes (Figure 7.1A). Formation of 2-PPA-CoA was rapid, reaching maximum concentration in 15 min in both control (1.1 nmol/million cells) and diabetic rats (4.1 nmol/million cells). At 3 h, the exposure of hepatic proteins to 2-PPA-CoA was 5-fold greater in diabetic rats than for controls (Figure 7.1A and Table 7.1). Diabetic rats also increased 2-PPA acyl glucuronidation 2 fold ($p < 0.05$) (Table 7.1). As shown in Figure 7.1B, 2-PPA acyl glucuronidation was roughly linear for 3 h, reaching 35 and 51

nmol/million cells respectively by the end of the incubations with hepatocytes from control and diabetic rats. As expected from the marked increase in 2-PPA-CoA formation and acyl glucuronidation (Figure 7.1A and B), a significant increase in covalent binding, up to 2.5 fold ($p < 0.001$), was observed in diabetic rats after 3 h incubation (Figure 7.1C).

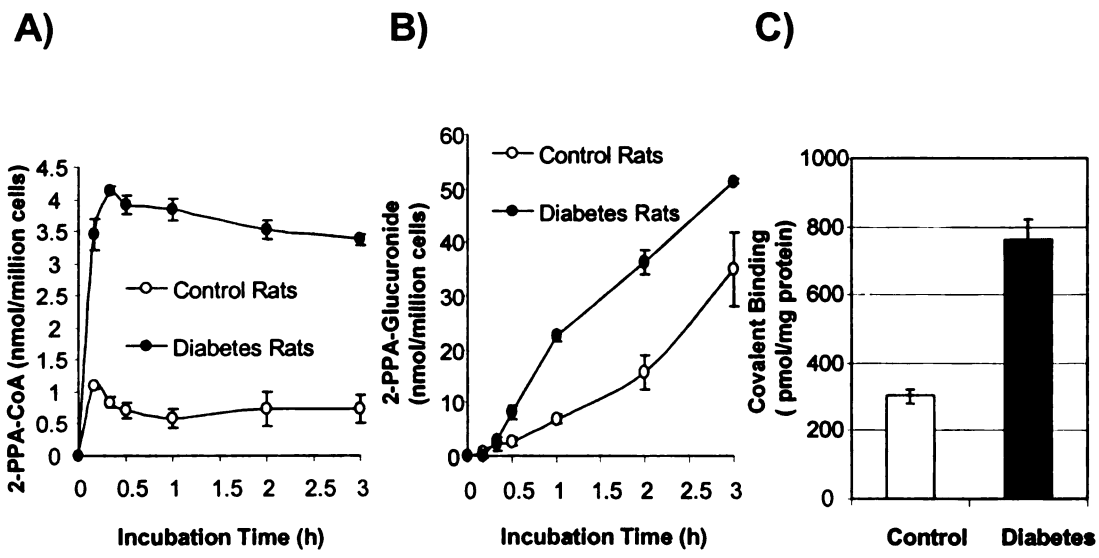


Figure 7.1 Representative time-course of the A) acyl-CoA formation, B) acyl glucuronidation and C) covalent binding of (*RS*)-[1-^{14}C]-2-PPA (1 mM) incubated with freshly isolated hepatocytes (4 million cells/mL) from streptozotocin induced diabetic and control rats. Values are expressed as the mean ± SD of triplicate incubations.

7.3.2 Effect of Diabetes on Enantiomeric Composition of 2-PPA and 2-PPA Acyl Glucuronides in Rat Hepatocytes

Like other widely-used profen drugs, such as ibuprofen and fenoprofen (Caldwell et al., 1988), the (*R*)-enantiomer of 2-PPA undergoes unidirectional chiral inversion to form the (*S*)-isomer (Yamaguchi and Nakamura, 1985). Enantioselective formation of (*R*)-2-PPA-CoA is believed to be the key step in mediating such chiral inversion. Since diabetic rats showed a marked increase in 2-PPA-CoA formation in rat hepatocytes, the effect of diabetes on the enantiomeric composition of 2-PPA and glucuronides was examined in the same incubations. Figure 7.2 shows the concentration-time curves for (*R*)- and (*S*)-2-PPA acids and their glucuronide metabolites in rat hepatocyte incubations. In control rats, both (*S*)-2-PPA acid and the glucuronide of the (*S*)-enantiomer showed significantly higher levels than those of the (*R*)-isomer. The *R/S* ratio of total 2-PPA (2-PPA plus glucuronide) decreased gradually with time in control rats from 1.0 at time 0 h to 0.73 at time 3 h after incubation with (*RS*)-2-PPA. In streptozotocin-induced diabetes the *R/S* ratio significantly decreased to 0.33 by selectively increasing the level of (*S*)-2-PPA and (*S*)-2-PPA-1-*O*-G and decreasing (*R*)-2-PPA in rat hepatocytes.

7.4 Discussion

Diabetes mellitus is known to cause profound alterations in hepatic fatty acid metabolism, which in part reflects an increase in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids. Enhanced hepatic fatty acyl-CoA synthesis is required for such an increase in hepatic utilization of fatty acids. Sharing the same cofactors (i.e., CoASH

Table 7.1 Effects of diabetes on 2-PPA-CoA formation, acyl glucuronidation and covalent binding *in vivo* in freshly isolated rat hepatocytes[a]

	$AUC_{0\text{-}3h}$ (nmol·h/million cells)		Covalent binding after 3h incubation (pmol/mg protein)
	2-PPA-CoA	2-PPA glucuronide	
Diabetes (D)	10.6 ± 0.1^b	82.0 ± 2.2^c	765 ± 58^b
Control (C)	2.09 ± 0.47	39.9 ± 8.0	301 ± 22
D/C Ratio	5.07	2.05	2.54

[a]Incubations of hepatocytes (4 million cells/ml) with (*RS*)-[1-^{14}C]-2-PPA (0.2 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, 10, 20, 30, 60, 120 and 180 min and analyzed for acyl-CoA formation and acyl glucuronidation. Values are expressed as the mean ± SD of triplicate incubations.
[b]$p < 0.001$ compared to the corresponding control group, using t test
[c]$p < 0.05$ compared to the corresponding control group, using t test

and ATP) and possibly the same or similar enzyme with fatty acyl-CoA synthesis, the formation of 2-PPA-CoA thioester was found to be significantly increased in streptozotocin-induced diabetic rats. As illustrated in Figure 7.1A and Table 7.1, there is a marked increase in hepatocellular exposure to 2-PPA-CoA (5-fold, $p < 0.001$) in streptozotocin-induced diabetic rats. An increase in acyl-CoA synthetase activity and hepatic availability of CoASH for acyl-CoA synthesis are potential mechanisms for mediating this enhancement of 2-PPA-CoA formation in diabetic rats.

Consistent with the marked increase in 2-PPA-CoA formation, a significant increase in metabolic chiral inversion of 2-PPA was observed in diabetic rat hepatocytes (Figure 7.2). As shown in Figure 7.2, streptozotocin-induced diabetes significantly increased the fractional inversion of (*R*)-2-PPA to the (*S*)-enantiomer by selectively increasing the level of (*S*)-2-PPA acid and (*S*)-2-PPA-1-*O*-G and decreasing (*R*)-2-PPA in rat hepatocytes.

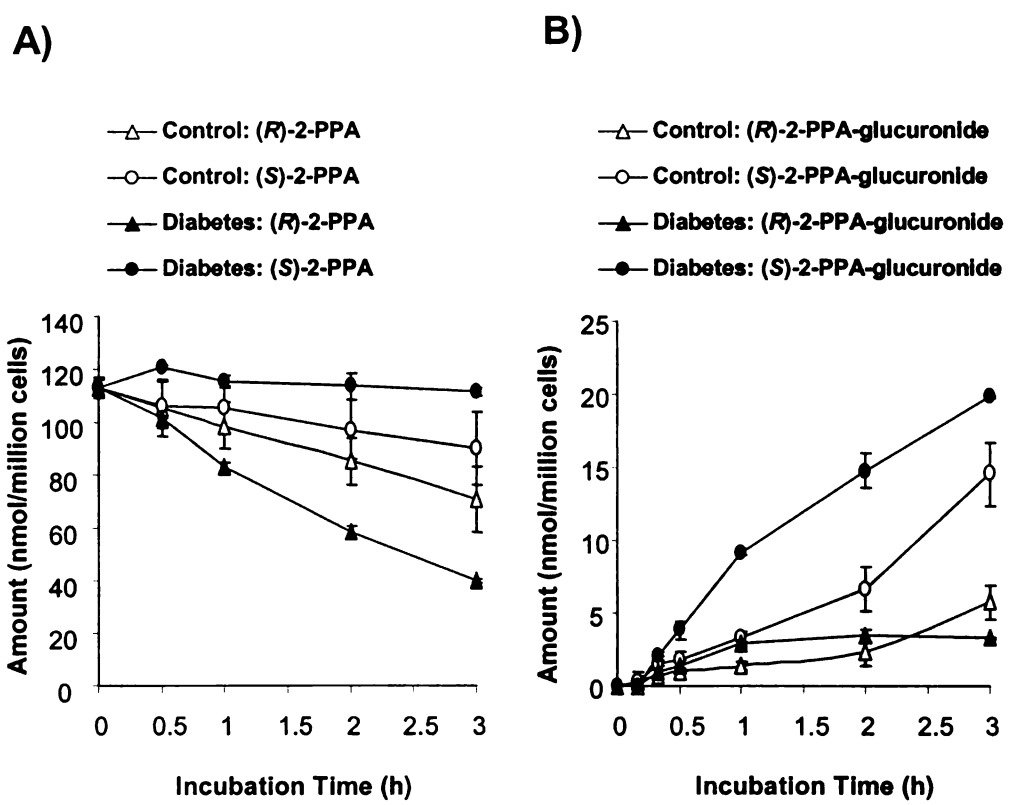
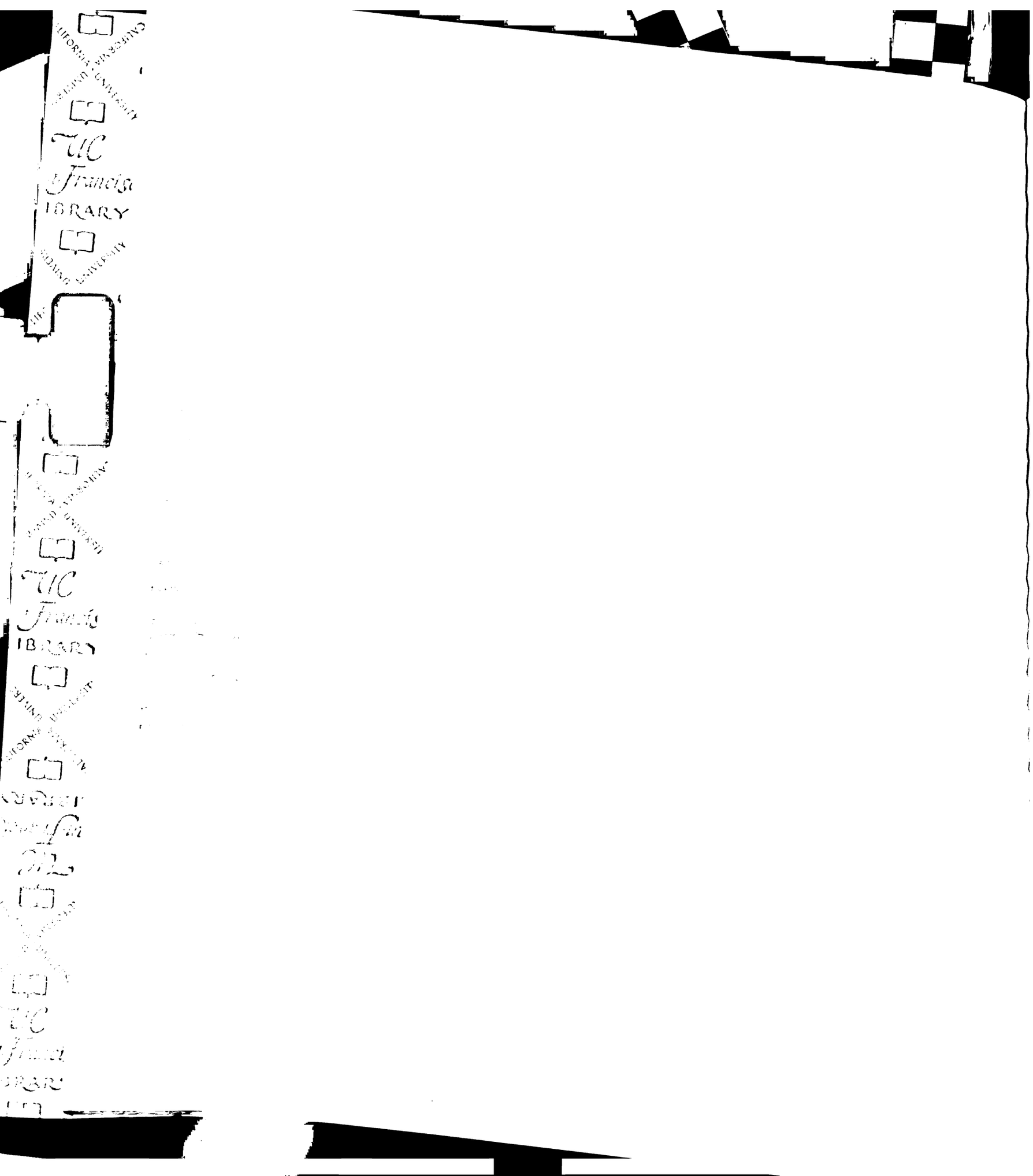


Figure 7.2 The effect of diabetes on enantiomeric composition of 2-PPA (A) and 2-PPA acyl glucuronides (B) in freshly isolated rat hepatocytes. Values are expressed as the mean ± SD of triplicate incubations.

In addition to a significant increase in acyl-CoA formation, diabetes mellitus also has a marked effect on glucuronidation. The change of glucuronidation in diabetic rats varies with substrate. A significant increase in glucuronidation of acetaminophen and *p*-nitophenol was demonstrated in diabetic rat hepatocytes (Price and Jollow, 1982; Eacho et al., 1981), whereas conjugation of 1-naphthol and phenolphthalein with glucuronic acid was reported to be markedly decreased in streptozotocin-induced diabetes (Grant



and Duthie, 1987). To date, there is little information on the effect of diabetes on glucuronidation of 2-arylpropionic acids. In the present study, we demonstrated that 2-PPA acyl glucuronidation in diabetic rats was 2 fold greater than that for controls ($p < 0.05$) (Figure 7.1B and Table 7.1). As expected from the marked increase in 2-PPA-CoA formation and acyl glucuronidation (Figure 7.1A and B), a significant increase in covalent binding of 2-PPA to hepatocyte proteins (2.5 fold, $p < 0.001$) was observed in diabetic rats after a 3 h incubation (Figure 7.1C).

In conclusion, the present studies demonstrate that streptozotocin-induced diabetes mellitus has marked effects on metabolic activation of 2-PPA. Both metabolic activation pathways, namely acyl-CoA formation and acyl glucuronidation, are significantly increased, resulting in a marked increase in irreversible binding of 2-PPA to hepatic proteins and possibly an increased risk of toxicity. Such an effect is likely to occur with other profens.

Chapter 8

Studies on the Enantioselective Acylation of Glutathione by (*R*)- and (*S*)-2-Phenylpropionic Acid Metabolites in Rats

8.1 Introduction

Acyl glucuronidation and acyl-CoA formation are common metabolic pathways for 2-arylpropionic acid drugs. In vitro studies with 2-PPA (a simple congener of 2-arylpropionic acid drugs) showed that both pathways were able to acylate the GSH sulfhydryl group to form the 2-PPA-*S*-acyl glutathione conjugate (2-PPA-SG, Figure 8.1) (Chapter 2 and Li et al., 2002). The resultant thioester glutathione derivative, a proposed detoxification product, is a polar, water-soluble metabolite that is either excreted unchanged into bile or into urine after further metabolism in the kidney. For those glutathione conjugates that are excreted into the urine, Grillo and Benet (2001) have recently demonstrated a new degradation pathway, which occurs to a much greater extent than degradation to the typical mercapturic acid conjugate (*S*-acyl-*N*-acetylcysteine conjugate). This novel urinary degradation pathway involves the initial cleavage of the γ-

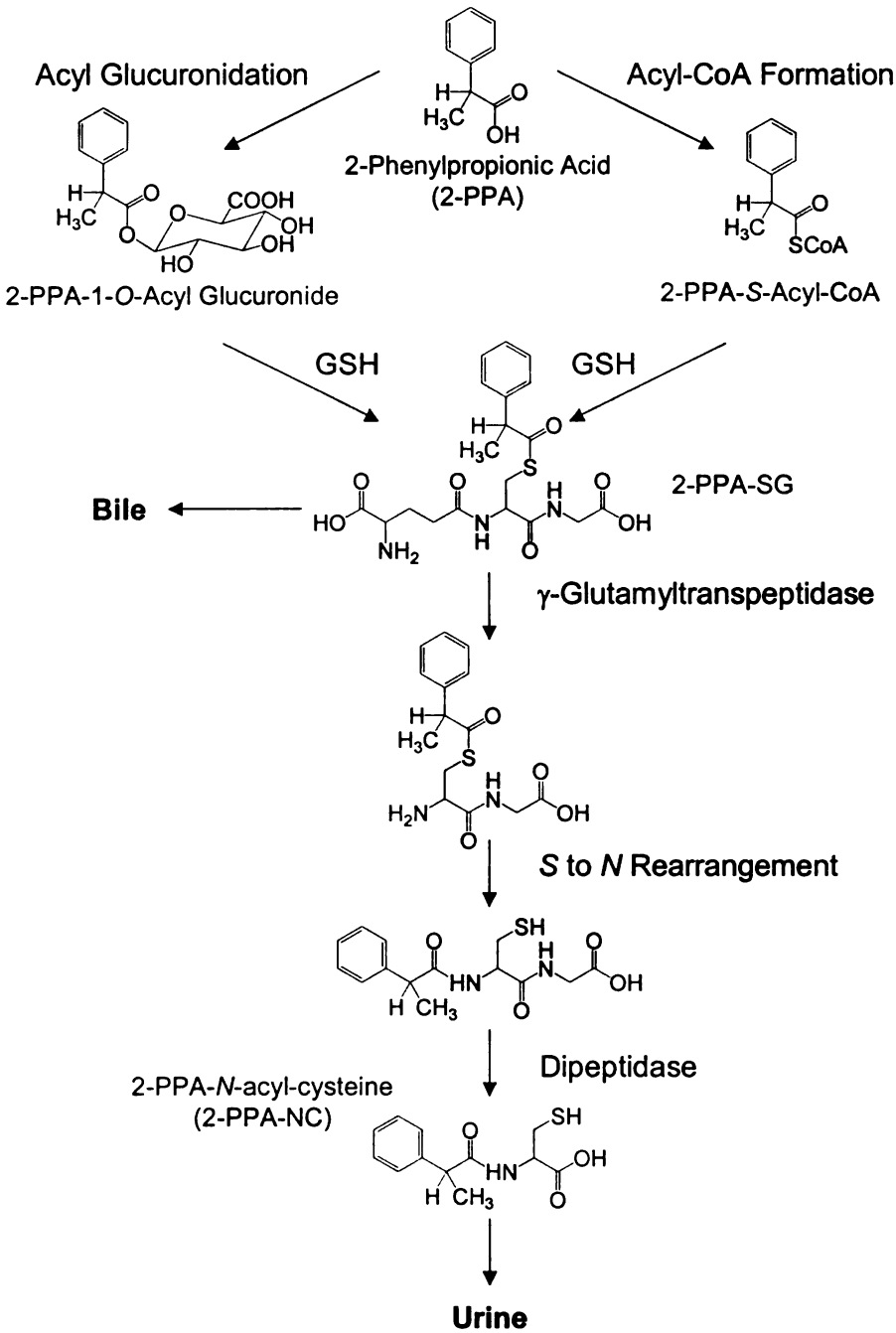


Figure 8.1 Proposed scheme for the formation and disposition of 2-PPA-*S*-acyl glutathione (2-PPA-SG) in vivo, which includes the two alternative metabolic activation pathways, transacylation of these reactive metabolites of 2-PPA with glutathione (GSH) and the excretion of 2-PPA-SG conjugates in bile unchanged and in urine as degradation end product, 2-PPA-*N*-acyl-cysteine (2-PPA-NC).

glutamyl portion of the conjugate by γ-glutamyltranspeptidase (γ-GT) leading to an *S*-acyl-cysteinylglycine product that quickly rearranges to the *N*-acyl-cysteinylglycine derivative (Figure 8.1). The resultant *N*-acyl-cysteinylglycine derivative was proposed to undergo further cleavage by dipeptidases in the kidney to the *N*-acyl-cysteine excretion product (Figure 8.1). Because the *N*-acyl-cysteine conjugate was shown to be a much more abundant urinary degradation product than the typical *S*-acyl-*N*-acetylcysteine conjugate (Grillo and Benet, 2001), the *N*-acyl-cysteine conjugate has been proposed as a urinary marker for *S*-acyl-glutathione conjugation in vivo (Figure 8.1).

In the present studies, we attempt to identify and quantify the *S*-acyl glutathione derivatives excreted into urine and bile from rats dosed with (*R*)- and (*S*)-enantiomers of 2-PPA and to determine which of the reactive metabolites could acylate GSH *in vivo* using an enantioselective approach. We demonstrate that metabolic activation by 2-PPA-CoA formation contributes more to the 2-PPA-SG formation than acyl glucuronidation in hepatocytes and in vivo in rats.

8.2 Experimental Section

8.2.1 Chemicals

(*R*)-2-PPA, (*S*)-2-PPA, (*RS*)-2-PPA, THF (anhydrous), trimethylamine, ethyl chloroformate (ECF) and perchloric acid (70%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Trifluoroacetic acid (TFA), $MgSO_4$, GSH and dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO). Ethylacetate, concentrated H_3PO_4 and potassium bicarbonate were purchased from Fisher Scientific (Fair Lawn,

NJ). Synthetic 2-PPA-CoA, 2-PPA-SG and biosynthetic 2-PPA-1-*O*-acyl glucuronide (2-PPA-1-*O*-G) were available from previous studies described in this thesis (Chapter 2 and Li et al., 2002). 2-PPA-*N*-acyl cysteine (2-PPA-NC) was synthesized as described below. All solvents used for HPLC were of chromatography grade.

8.2.2 Synthesis of 2-PPA-NC derivative

Synthesis of 2-PPA-NC was performed by conventional procedures employing ECF (Stadtman, 1957). Briefly, to 2-PPA (240 mg, 1.6 mmol) dissolved in anhydrous THF (25 ml), triethylamine (220 µl, 1.6 mmol) followed by ECF (100 µl, 1.6 mmol) was added, at room temperature and while stirring. After 30 min, a white precipitate (triethylamine hydrochloride) was formed and removed by passage through a glass funnel fitted with a glass wool plug directly into a solution containing cysteine (200 mg, 1.6 mmol) and $KHCO_3$ (160 mg, 1.6 mmol) in distilled water (10 ml) and THF (15 ml). After 2 h stirring under nitrogen gas at room temperature, the reaction was terminated by the addition of concentrated HCl (10 drops, pH~3). The THF was then removed by evaporation under reduced pressure and the remaining aqueous phase was adjusted to pH 7.0 by the addition of NaOH (1M). Purification of 2-PPA-NC was achieved by reverse-phase HPLC using isocratic elution with 40% methanol in 0.05 M potassium phosphate buffer (pH 5.7). A Microsorb-MV C_{18} column (150 x 4.6 mm, 5 µm particle size, Varian Analytical Instruments, Walnut Creek, CA) was used at a flow rate of 1 ml/min and with UV detection at 226 nm. 2-PPA-NC containing fractions were collected and desalted by passing through Sep Pak C_{18} cartridges (Varian Inc., Harbor City, CA). Desalted 2-PPA-NC was dried under a stream of nitrogen and stored at -20°C. The identity of 2-PPA-NC

was confirmed by ESI/MS (Hewlett Packard HPLC 1100LC/MSD bench-top mass spectrometer) operating in positive ion mode: *m/z* 254 (MH^+, 30%) and 105 ($[C_6H_5CH(CH_3)]^+$, 100%).

8.2.3 Animals

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

8.2.4 In Vivo Studies with (*R*)-2-PPA and (*S*)-2-PPA

Tissue Collection. Rats were treated with (*R*)-2-PPA or (*S*)-2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat) by intraperitoneal (i.p.) injection. After 2-PPA administration, rats were killed at 0, 5, 10, 20, 60, 120 and 180 min and their livers removed and immediately frozen in liquid nitrogen. Livers were stored at -80°C for the analysis of 2-PPA-CoA formation and acyl glucuronidation, using the methods described previously (Chapter 5). Two rats were utilized for each time point for (*R*)- and (*S*)-2-PPA-treated groups.

Urine Collection. Rats (n = 4 for each group) were given a single dose of (*R*)-2-PPA or (*S*)-2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat, i.p.). Sixteen hours post

administration, during which time the animals were kept unrestrained in metabolic cages, total urines (20-25 ml) were collected, acidified (pH 2.5, concentrated H_3PO_4), and extracted with equal volumes of ethylacetate (3 times). The combined extracts were dried with $MgSO_4$ and evaporated to dryness with N_2 gas at room temperature. Residues were dissolved in 50% acetonitrile and vortex mixed. Portions (100 μL) of dissolved extracts were mixed with DTT ((1 mM final concentration) for 30 min and analyzed by reverse-phase gradient LC/MS/MS. DTT is used to reduce any disulfide bond in rat urine extract to the free cysteinyl sulfhydryl form.

Bile Collection. Rats (n = 4 for each group) were anesthetized with ketamine/xylazine and their bile ducts were cannulated with PE-10 tubing. The cannulated rats were given a single dose of (*R*)-2-PPA or (*S*)-2-PPA (130 mg/kg in 0.9% saline, 0.5 ml/rat) and biles (7~ 9 mL) were collected over 0.5 ml of concentrated phosphoric acid for 6 h post 2-PPA dosing. Aliquots of the acidified rat bile were mixed with an equal volume of acetonitrile and precipitates were removed via centrifugation at 10,000 *g* for 5 min. The resultant supernatants were analyzed by LC/MS/MS.

8.2.5 In Vitro Studies with Rat Hepatocytes

Freshly-isolated rat hepatocytes were prepared from one rat (300 g, male Sprague-Dawley) according to published procedures (Moldeus et al., 1978). Viability greater than 85% was achieved, as assessed by trypan blue exclusion. Incubations of hepatocytes (4 million cells/ml) with (*R*)- or (*S*)-2-PPA (1.0 mM) were performed in Krebs-Henseleit buffer (pH 7.4) in 25 mL round bottom flasks with continuous rotation and gassed with 95% O_2/5% CO_2 at 37°C. Aliquots of the incubation (0.5 mL) were taken at 0, 5,10, 20

and 40 min and quenched with 0.1% TFA in acetonitrile (0.5 mL). After centrifugation (10,000 *g*, 10 min), the resulting supernatants were analyzed by LC/MS/MS in the positive ion mode, and by selected reaction monitoring (SRM) analysis of 2-PPA-SG formation with the MH^+ *m/z* 440 transition to the *m/z* 208 fragment.

8.2.6 Instrumentation and Analytical Methods

HPLC analysis was carried out on a Shimadazu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadazu Corp., Japan) with a Shimadazu SCL-10A controller and a Shimadazu SPD-10A UV-Vis detector. Determination of 2-PPA-CoA formation and acyl glucuronidation in rat livers was carried out by reverse-phase HPLC, as described previously (Chapters 4 and 5). Quantitative measurements of 2-PPA-CoA and 2-PPA-acyl glucuronide formation were made using a standard curve by adding liver samples of untreated rats with synthetic 2-PPA-CoA or biosynthetic 2-PPA-1-*O*-G, followed by the processes described above. Electrospray LC-MS and LC-MS/MS of synthetic standards and in vitro biological extracts was performed on a Hewlett Packard HP 1100 LC/MSD bench-top electrospray mass spectrometer and a Finnigan-MAT TSQ 7000 (San Jose, CA), respectively. Analysis of 2-PPA-SG conjugate in hepatocytes and bile and rearranged degradation product (2-PPA-NC) in reconstituted urine extracts was conducted by LC/MS/MS, with gradient elution from 5% to 100% acetonitrile over 20 min in 0.1% TFA on a Zorbax C18 column (150 x 2.1 mm, 5 μ, MAC-MOD Analytical, Chadds Ford, PA) at a flow rate of 0.3 ml/min. LC/MS/MS was carried out in positive ion mode, and by selective reaction monitoring (SRM). The amounts of 2-PPA-SG in hepatocytes and bile, and 2-PPA-NC in

urine were quantified using a standard curve by spiking hepatocytes, blank bile and urine with synthetic 2-PPA-SG or 2-PPA-NC, followed by the processes described above.

8.3 Results

8.3.1 Enantioselective Metabolism of (*R*)- and (*S*)-2-PPA in Rat Liver

After administration of (*R*)- and (*S*)-2-PPA (130 mg/kg, i.p.) to rats, the formation of 2-PPA acyl glucuronide, as indicated by the amount in rat liver tissue, was selective for the (*S*)-isomer (*R/S* = 0.6), whereas formation of 2-PPA-CoA thioester is selective for the (*R*)-enantiomer (*R/S* = 7.5), which is consistent with the results from previous hepatocyte studies (Chapter 4). As shown in Figure 8.2, the concentration of 2-PPA acyl glucuronide in the liver was much greater than that of 2-PPA-CoA thioester, indicating the relatively higher capacity of acyl glucuronidation.

8.3.2 Enantioselective Acylation of GSH by (*R*)- and (*S*)-2-PPA in Vivo in Rats

Once formed in the liver, 2-PPA-SG conjugate undergoes sequential degradation steps to the rearranged degradation product, 2-PPA-NC derivative, which is eventually excreted into urine (Figure 8.1). In order to identify and quantify the rearranged degradation product, rat urine (20-25 ml) was collected over sixteen hours post administration of (*R*)- or (*S*)-2-PPA (130 mg/kg, i.p.). LC/MS/MS analysis of the processed urine extracts by SRM in the positive mode using the transition MH^+ *m/z* 254 to *m/z* 105 showed the presence of the 2-PPA-NC derivative in urine from rats dosed with

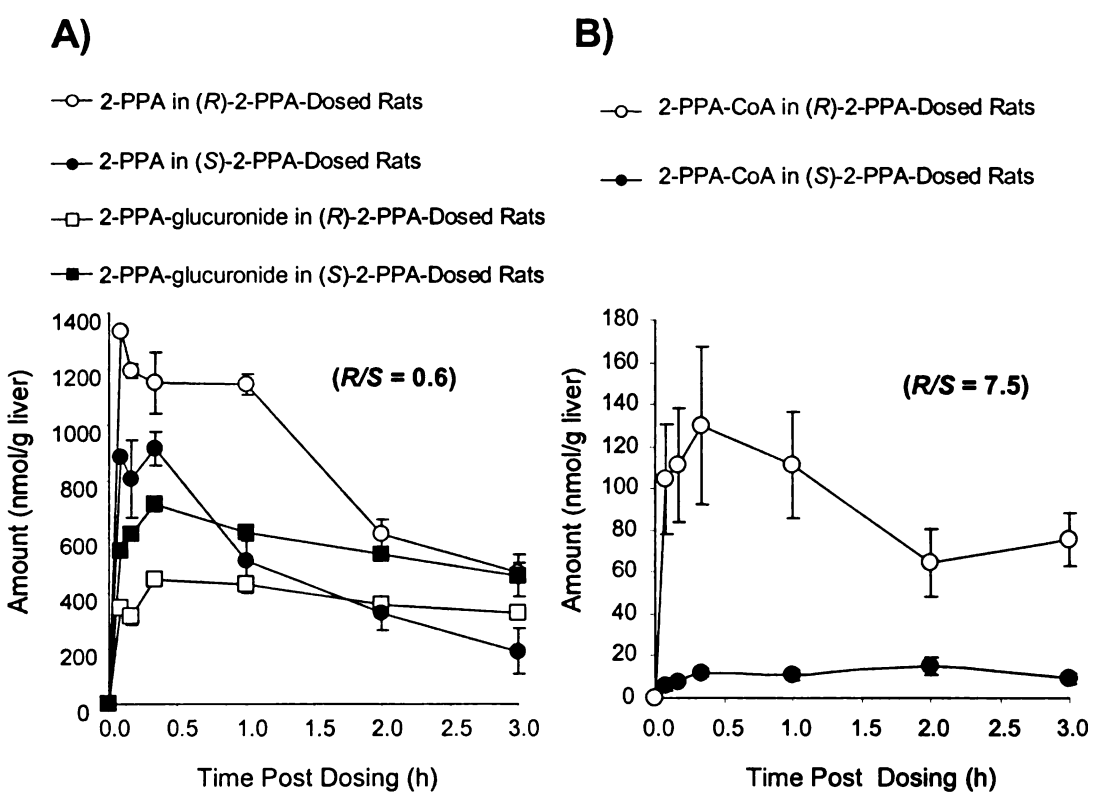


Figure 8.2 Time-dependent 2-PPA acyl glucuronidation (A) and acyl-CoA formation (B) in livers from rats dosed with (*R*)-2-PPA or (*S*)-2-PPA (130 mg/kg, i.p.). Values are expressed as the mean ± SD (n = 2 at each time point).

(*R*)- or (*S*)-2-PPA. As shown in Figure 8.3, the (*R*)- and (*S*)-2-PPA-NC derivatives were separable, with the (*R*)-isomer eluting later than (*S*)-antitope. Urine extracts from untreated-rats upon LC/MS/MS analysis exhibited no peaks eluting at the retention times of 2-PPA-NC (data not shown). Administration of (*R*)-2-PPA to rats led to the excretion of both (*R*)- and (*S*)-*N*-acyl-cysteine products, indicating chiral inversion, whereas only the (*S*)-isomer was detected post (*S*)-2-PPA administration (Figure 8.3). The identities of these products were confirmed by comparing tandem mass spectra to authentic synthetic

standards. The amount of 2-PPA-NC conjugate detected in 16 h rat urine extracts indicted that rat dosed with (*R*)-isomer (16.7 nmol) excreted 4.1 times as much of the derivative as those in (*S*)-2-PPA dosed rats (4.1 nmol; $p < 0.05$) (Figure 8.4).

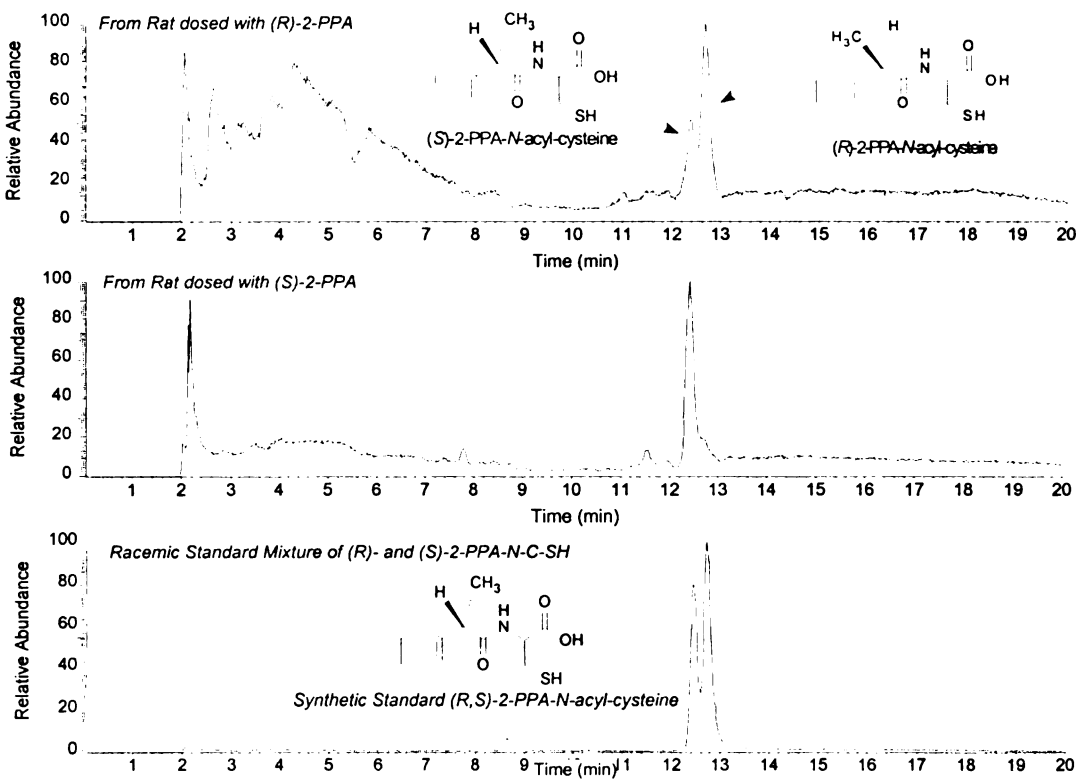


Figure 8.3 Representative reverse-phase gradient LC/MS/MS SRM chromatogram of 2-PPA-*N*-acyl-cysteine (2-PPA-NC) conjugate in urine extracts from rats dosed with (*R*)- and (*S*)-2-PPA (130 mg/kg, i.p.).

In addition to excretion into urine as 2-PPA-NC conjugate, 2-PPA-SG could also be excreted into bile unchanged (Figure 8.1). To obtain the total formation of 2-PPA-SG in vivo in rats, rat bile (7-9 ml) was collected and processed after administration of (*R*)-

or (*S*)-2-PPA (130 mg/kg) to bile duct-cannulated rats. The analysis of 2-PPA-SG in the processed bile samples was carried out by LC/MS/MS in the positive mode using SRM of the transition MH^+ *m/z* 440 to *m/z* 208, which showed the presence of 2-PPA-SG in bile from rats treated with (*R*)- or (*S*)-2-PPA. No 2-PPA-SG thioester was detected in bile samples from untreated rats. The identities of 2-PPA-SG were confirmed by comparing tandem mass spectra to authentic synthetic standard. As shown in Figure 8.4, (*R*)-2-PPA-dosed rats excreted somewhat more 2-PPA-SG conjugate into rat bile than (*S*)-2-PPA-dosed rats (*R/S* = 1.9). The amount of 2-PPA-SG excreted into bile over 6 h was significantly less than the derived conjugates excreted into urine over 16 h for both (*R*)- and (*S*)-2-PPA-dosed rats (Figure 8.4). Preliminary studies showed that more than 90% of 2-PPA-SG thioester in the bile was recovered during the first 6 h of bile collection. Due to the difficulty in maintaining a constant bile flow after the first 6 h collection, 6-h bile collection was therefore utilized in the present studies. The total formation of 2-PPA-SG in vivo in rats, as indicated by the total amount of *S*-acyl glutathione derivatives excreted into urine and bile, was enantioselective for the (*R*)-isomer (*R/S* = 3.3, Figure 8.4), which was better correlated with the enantioselectivity of acyl-CoA formation (*R/S* = 7.5) than acyl glucuronidation (*R/S* = 0.6) in (*R*) and (*S*)-2-PPA dosed rats.

8.3.3 Hepatocyte studies with (*R*)- and (*S*)-2-PPA

Having demonstrated the enantioselective formation of 2-PPA-SG in vivo in rat, we performed studies to confirm such enantioselectivity in freshly isolated hepatocytes. As shown in previous studies (Chapter 4), the conversion of 2-PPA to the acyl

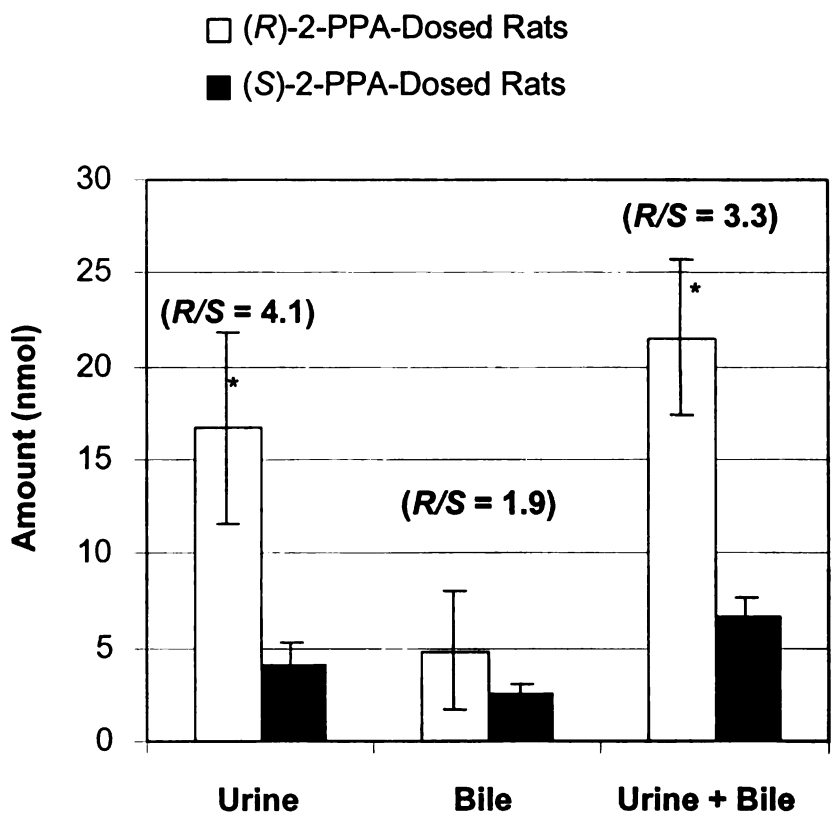


Figure 8.4 Excretion of 2-PPA-SG derived conjugates into urine (16 h) and bile (6 h) from rats dosed with (*R*)- or (*S*)-2-PPA (130 mg/kg). Values are expressed as the mean ± SD (n = 4).

glucuronide is enantioselective for the (*S*)-enantiomer (*R/S* = 0.67), whereas acyl-CoA formation of 2-PPA is enantioselective for the (*R*)-enantiomer (*R/S* = 7.0) in rat hepatocytes. In the present studies, we found that the formation of 2-PPA-SG was 6.0 fold greater for the (*R*)-2-PPA isomer (0.189 pmol/million cells) than the (*S*)-isomer (0.031 pmol/million cells) after 3 h incubation with rat hepatocytes (Figure 8.5). This enantioselective acylation of GSH by (*R*)- and (*S*)-2-PPA correlated with the enantioselective formation of 2-PPA-CoA in incubations with hepatocytes (Chapter 4).

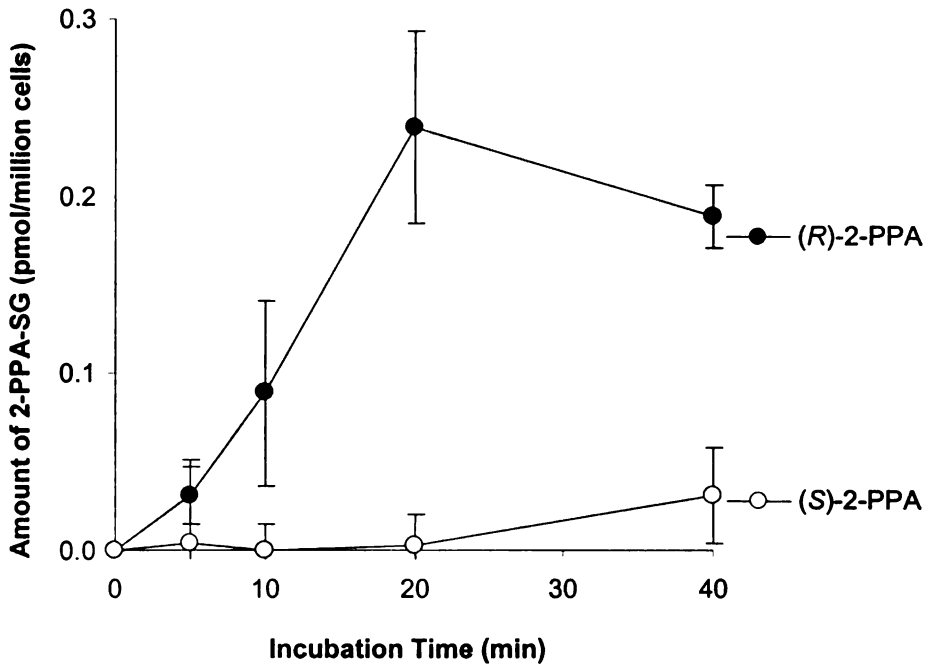


Figure 8.5 Enantioselective formation of 2-PPA-SG in incubations of (*R*)- or (*S*)-2-PPA (1 mM) with freshly isolated hepatocytes (4 million cells/ml). Values are expressed as the mean ± SD of triplicate incubations.

8.4 Discussion

Most 2-arylpropionic acids are metabolized by two metabolic pathways, namely acyl glucuronidation and acyl-CoA formation. Both pathways form reactive metabolites that may lead to covalent binding to proteins and thus potentially contribute to the idiosyncratic hepatotoxicity of 2-arylpropionic acid drugs (Boelsterli, 2002). Alternatively, these reactive intermediates could be scavenged by their reaction with GSH. Conjugation with GSH potentially protects protein nucleophiles from reactive

metabolites of 2-arylpropionic acids and therefore plays an important role in the detoxification of metabolic activation of 2-arylpropionic acids. In vitro studies with 2-PPA showed that both metabolic pathways, namely acyl-CoA thioester formation and acyl glucuronidation, were able to acylate the GSH sulfhydryl group to form 2-PPA-SG (Chapter 2 and Li et al., 2002). 2-PPA-*S*-acyl-CoA (2-PPA-CoA) was approximately 70-fold more reactive towards GSH than the respective acyl glucuronide in buffer solution (Li et al., 2002). However, the relative importance of these two metabolic pathways for 2-PPA-SG formation in the in vivo situation remained unresolved. In the present studies, we attempted to identify the major metabolic activation pathway of 2-PPA that leads to the formation of acyl glutathione in vivo in rats, by using an enantioselective approach.

As shown in Figure 8.1, after conversion to the acyl glucuronide or acyl-CoA thioester, 2-PPA reacts with GSH to form the polar and water soluble product, 2-PPA-SG, which is excreted both unchanged in bile and after sequential enzyme-catalyzed degradation steps to the *N*-acylcysteine conjugate in the urine. LC/MS/MS SRM analyses showed 2-PPA-SG in bile and 2-PPA-NC in urine from rats dosed with either (*R*)- or (*S*)-2-PPA. The identities of these products were confirmed by comparing tandem mass spectra to authentic synthetic standards. LC/MS/MS analysis showed that the rearranged γ-GT/dipeptidase product, 2-PPA-NC, was the only observable glutathione-derived conjugate in rat urine. No mercapturic acid conjugate was detected, consistent with previous studies with clofibric acid (Grillo and Benet, 2001).

2-PPA undergoes chiral inversion from the (*R*)- to the (*S*)-enantiomer via an acyl-CoA thioester intermediate (Fournel and Caldwell, 1986). The formation of 2-PPA-CoA thioester is enantioselective for the (*R*)-enantiomer (Figure 8.2), whereas acyl

glucuronidation is selective for the (*S*)-2-PPA enantiomer (Figure 8.2). Both acyl-CoA thioester and acyl glucuronide could transacylate glutathione forming 2-PPA-SG in vitro (Chapter 2 and Li et al., 2002) and thus are potentially involved in formation of 2-PPA-SG derivatives in vivo in rats dosed with 2-PPA. We predict that these enantiomeric differences in metabolism of (*R*)- and (*S*)-2-PPA isomers determine the enantioselectivity of 2-PPA-SG formation in vivo and the total excretion of 2-PPA-SG derived conjugates in urine and bile. If metabolic activation by 2-PPA-CoA formation is important, excretion of 2-PPA-SG derived conjugates into urine and bile should be greater from rats dosed with (*R*)- than with (*S*)-2-PPA isomer. Conversely, the (*S*)-2-PPA-treated rats would excrete more 2-PPA-SG derived conjugates into urine and bile if acyl glucuronidation is the most important. Our in vivo studies with (*R*)- and (*S*)-enantiomers showed that the total formation of 2-PPA-SG in vivo in rats, as indicated by the total amount of *S*-acyl glutathione derivatives excreted into urine and bile, was enantioselective for the (*R*)-isomer (*R/S* = 3.3). Such enantioselectivity of 2-PPA-SG formation correlated better with the enantioselectivity of acyl-CoA formation (*R/S* = 7.5) than with the enantioselectivity of acyl glucuronidation (*R/S* = 0.6) of (*R*)- and (*S*)-2-PPA enantiomers. These results strongly suggest that acyl-CoA derivatives play a more important role in 2-PPA-SG formation in vivo in rats than the respective acyl glucuronide metabolites, even though the formation of 2-PPA-acyl glucuronides was much greater than 2-PPA-CoA formation in the liver (Figure 8.2). We believe that the higher contribution of the acyl-CoA pathway to 2-PPA-SG formation results from the higher chemical reactivity of acyl-CoA thioesters towards the GSH sulfhydryl group, which is consistent with our recent in vitro studies with GSH (Chapter 2 and Li et al., 2002).

Having demonstrated the enantioselective formation of 2-PPA-SG in vivo, we performed further studies in freshly isolated hepatocytes. As shown in previous studies (Chapter 4), the conversion of 2-PPA to the acyl glucuronide is enantioselective for the (*S*)-enantiomer (*R/S* = 0.67), whereas acyl-CoA formation of 2-PPA is enantioselective for the (*R*)-enantiomer (*R/S* = 7.0) in rat hepatocytes. Such enantiomeric ratios are very similar to those observed in vivo in rats (Figure 8.2). Figure 8.5 shows the time-dependent formation of 2-PPA-SG in hepatocyte incubations with (*R*)- and (*S*)-enantiomers. The formation of 2-PPA-SG was 6.0 fold greater after 3 h of incubation with (*R*)-2-PPA (0.189 pmol/million cells) than with (*S*)-2-PPA (0.031 pmol/million cells), which correlates with the enantioselectivity of acyl-CoA formation, but not with acyl glucuronidation of the (*R*)- and (*S*)-2-PPA-isomers. These results further confirm conclusions from our in vivo enantioselective studies and strongly indicate that freshly isolated hepatocytes are a good in vitro system to study the metabolic activation of xenobiotics.

Recently, we demonstrated that covalent binding of 2-PPA to protein was enantioselective for the (*R*)-enantiomer in hepatocyte incubations with the (*R*)- and (*S*)-2-PPA isomers (Chapter 4), consistent with the enantioselectivity of 2-PPA-SG formation observed here. Hepatocyte studies with (*R*)- and (*S*)-2-PPA enantiomers showed that the extent of covalent binding is 4.5 fold greater after 3 h of incubation with (*R*)-2-PPA than with (*S*)-2-PPA, whereas the ratio of 2-PPA-SG formation in hepatocytes is *R/S* = 6.0. Since acyl glutathione conjugation is closely associated with covalent adduct formation in hepatocytes, we propose that acyl glutathione could serve as a soluble marker for the covalent binding to hepatic tissue protein in vivo. Since it is difficult to obtain human

liver samples to study the extent of hepatic covalent binding, measurement of 2-PPA-SG excreted into urine and bile could serve as an alternative, non-invasive method.

In summary, results from these studies suggest that metabolic activation of 2-PPA by acyl-CoA formation provides more reactive acylating species in the liver than metabolic activation by acyl glucuronidation. We propose that acyl-CoA thioester derivatives of carboxylic acid-containing drugs, besides acylating glutathione, contribute to the acylation of hepatic protein nucleophiles in vivo and possibly to allergic reactions associated with the use of certain acidic drugs.

Chapter 9

In Vitro Studies on the Chemical Reactivity of 2,4-Dichlorophenoxyacetyl-*S*-acyl-CoA Thioester

9.1 Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D), a chlorophenoxy herbicide, has been extensively used in the control of broadleaf and woody plants. The herbicide acts as a synthetic plant hormone, altering the plant's metabolism and hence growth characteristics (Stevens and Summer, 1991). Human and animal exposure to 2,4-D through agricultural use, food products, or through use in lawn and garden care has been well documented (Taskar et al., 1982; Harris and Solomon, 1991; Reynolds et al., 1993). The effect of such exposure to mammal health, however, has not yet been completely assessed. In incubations with freshly-isolated rat hepatocytes, 2,4-D has been shown to induce time- and dose-dependent cell death accompanied by rapid depletion of intracellular glutathione (GSH) (Palmeira et al., 1994; Palmeira et al., 1995). Elevation of serum alanine and aspartate aminotransferases, indicators of hepatic injury, was observed in rats chronically treated with 2,4-D (Charles et al., 1996). Histological and histochemical

[1]This chapter will be published in part as a manuscript entitled "*In Vitro* Studies on the Chemical Reactivity of 2,4-Dichlorophenoxyacetyl-*S*-acyl-CoA Thioester" Li C, Grillo MP, Benet LZ, *Toxicol. Appl. Pharmacol.* in press. Reproduced with permission.

changes in livers of newborn and adult rats were also demonstrated when rats were administrated 2,4-D during their prenatal and postnatal periods (Sulik et al., 1998). These animal studies clearly indicate that liver toxicity appears to be one of the major toxic effects associated with the exposure to 2,4-D. Furthermore, "Golf ball liver", an acute hepatitis, has been reported to be directly related to exposure to 2,4-D by golfers who inadvertently ingested the agent (Leonard et al., 1997).

Currently, the mechanisms underlying 2,4-D-induced liver toxicity are poorly understood. Covalent modification of liver proteins by reactive metabolites of 2,4-D could be involved, as suggested by mechanistic studies of drug-induced hepatitis (Pumford and Halmes, 1997). Several reports have demonstrated that 2,4-D binds irreversibly to hepatic proteins in rat and chick (Kelley and Vessey, 1987; Evangelista de Duffard et al., 1993; Di Paolo et al., 2001). Recently Di Paolo *et al.* (2001) identified a 52-kDa protein modified selectively by 2,4-D in rat mitochondrial preparations both in vitro and in vivo. However, the reactive metabolites of 2,4-D that mediate such covalent binding have not been identified. The aim of the present study was to investigate the chemical reactivity of 2,4-dichlorophenoxyacetyl-*S*-acyl-CoA (2,4-D-CoA) and to determine its potential involvement in 2,4-D covalent binding to protein.

Acyl-CoA thioesters of xenobiotic carboxylic acids (Fears, 1985) serve as necessary intermediates for the formation of amino acid conjugates, acyl carnitine and choline derivatives (Figure 9.1). The activation of endogenous fatty acids to their

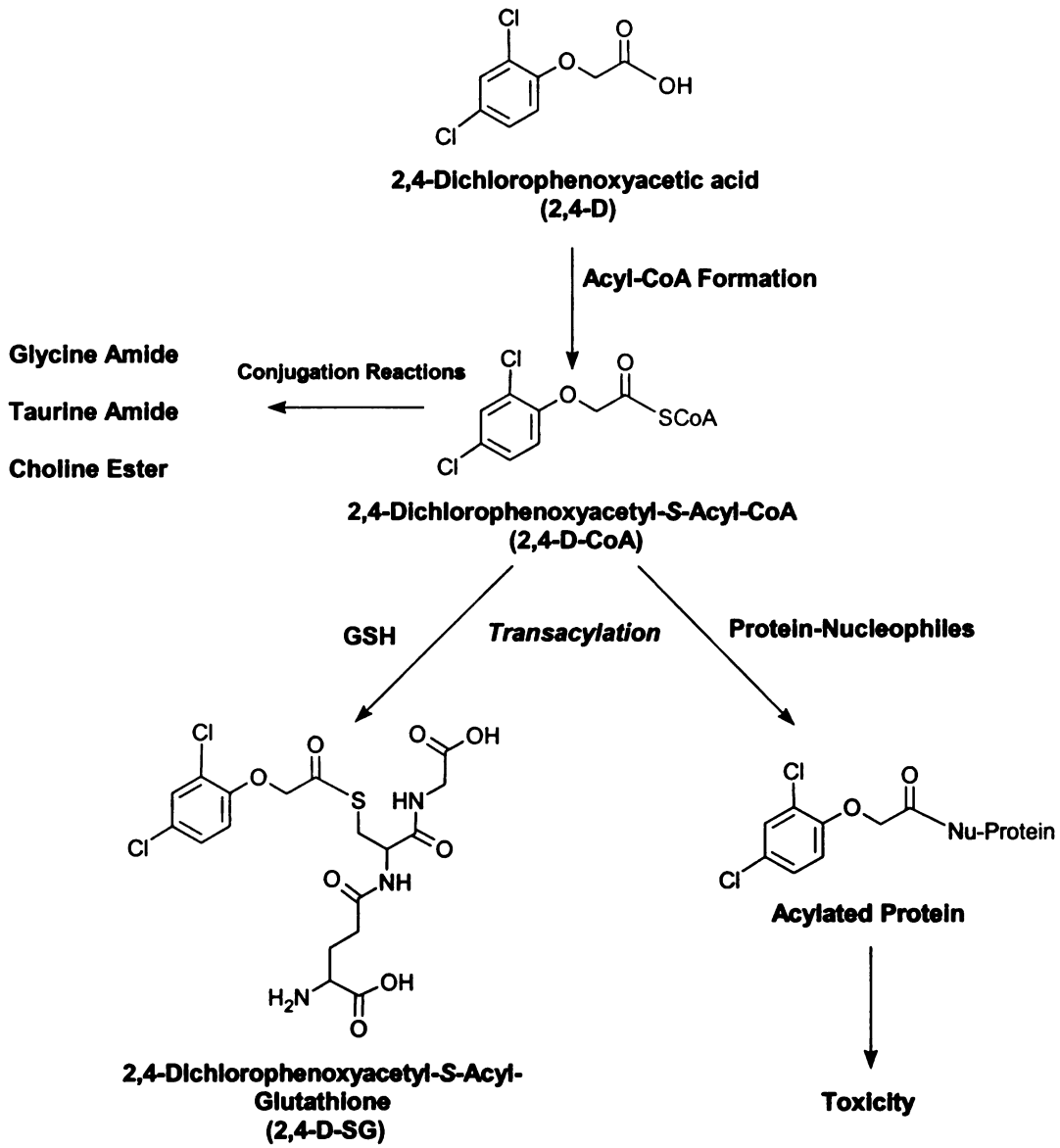


Figure 9.1 Schematic representation of the potential metabolic fate of 2,4-D-CoA thioester.

corresponding high-energy acyl-CoA thioesters is required for their cellular usage, such as fatty acid synthesis and degradation, triacylglyceride formation, and protein fatty acylation. All of these processes require a reactive thioester bond, and therefore xenobiotic acyl-CoA derivatives are also believed to be electrophilic in nature. Sallustio *et al.* (2000) showed that covalent binding of nafenopin to human liver proteins is directly associated with the formation of a nafenopin acyl-CoA thioester intermediate.

Studies on the biological fate of 2,4-D in vivo in rats revealed that 2,4-D is primarily excreted as parent compound along with taurine and glycine conjugates (Griffin et al., 1997a; Griffin et al., 1997b). 2,4-D-CoA thioester is believed to be the high energy intermediate necessary for both conjugation reactions. A study in rat liver microsomes provided direct evidence of the ability of rat liver to catalyze the formation of 2,4-CoA thioester (Sastry et al., 1997).

Our recent studies have shown that 2-phenylpropionyl-*S*-acyl-CoA (2-PPA-CoA) is a reactive electrophile that is able to transacylate the GSH sulfhydryl group and protein nucleophiles (Chapter 2 and Li et al., 2002). Therefore, we wondered whether 2,4-D-CoA is reactive enough to acylate protein and other nucleophiles. In the present studies we investigated the reactivity of 2,4-D-CoA with GSH and human serum albumin (HSA), and the irreversible binding of 2,4-D to proteins in incubations with rat hepatocytes. We find that an inhibitor of acyl-CoA formation significantly decreased the extent of 2,4-D covalent binding to protein, indicating that 2,4-D-CoA most likely mediated the formation of 2,4-D-protein covalent adducts detected in 2,4-D-treated hepatocytes.

9.2 *Experimental Section*

9.2.1 Chemicals

[1-^{14}C]-2,4-D, phenylacetyl-*S*-acyl CoA (PAA-CoA), CoA, ammonium acetate, human serum albumin (HSA, ≥ 96% albumin, essential fatty acid free) and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4-D, PAA, 2-PPA, perchloric acid (70%), and [(1*S*)-*endo*]-(-)-borneol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diethyl ether and potassium phosphate monobasic were from Fisher Scientific (Fair Lawn, NJ). [1,2-^{14}C]-PAA was purchased from Moravek Biochemicals (Brea, CA). Hionic-Fluor scintillation fluid was purchased from Packard BioScience Co. (Meriden, CT). Authenic 2,4-D-*S*-acyl glutathione (2,4-D-SG) was available from prior syntheses in our laboratory (Grillo and Benet, 2001). Synthetic 2-PPA-*S*-acyl glutathione (2-PPA-SG) and 2-PPA-CoA were obtained from previous studies described in this thesis (Chapter 2 and Li et al., 2002). 2,4-D-CoA, [1-^{14}C]-2,4-D-CoA, [1,2-^{14}C]-PAA-CoA, and PAA-*S*-acyl glutathione (PAA-SG) were synthesized as described below. All solvents used for HPLC analysis were of chromatographic grade.

9.2.2 Synthesis of 2,4-D-CoA, [1-^{14}C]-2,4-D-CoA and [1,2-^{14}C]-PAA-CoA

The synthesis and purification of acyl-CoA thioesters of 2,4-D, [1-^{14}C]-2,4-D and [1,2-^{14}C]-PAA were performed by conventional procedures employing ethyl chloroformate, as reported previously for the synthesis and purification of clofibryl-*S*-acyl-CoA thioester (Grillo and Benet, 2001). The identity of 2,4-D-CoA was confirmed using a Matrix-Assisted Laser Desorption Ionization (MALDI) time-of-flight instrument (PerSeptive Biosystems, Voyager Elite mass spectrometer, Framingham, MA) equipped

with a nitrogen laser (337 nm). Mass spectra for 2,4-D-CoA, *m/z*: 1008 ($[M + K]^+$), 992 ($[M + Na]^+$), 970 ($[M + H]^+$), 463 ([M + H- adenosine triphosphate]$^+$), 428 ([adenosine diphosphate + 2H]$^+$), and 334 ($[2,4\text{-}Cl_2\text{-}C_6H_3OCH_2COS(CH_2)_2NHCO(CH_2)_2]^+$). [1,2-^{14}C]-PAA-CoA was confirmed by coelution with commercially available standard PAA-CoA. Concentrations of the synthetic 2,4-D-CoA, [1-^{14}C]-2,4-D-CoA and [1,2-^{14}C]-PAA-CoA were determined by quantification of the free acid released after alkaline hydrolysis. Reverse-phase HPLC followed by scintillation counting of HPLC fractions, collected every 30 sec, showed that the radiochemical purities of [1,2-^{14}C]-PAA-CoA and [1-^{14}C]-2,4-D-CoA were both 100 %.

9.2.3 Synthesis of PAA-*S*-Acyl Glutathione (PAA-SG)

The synthesis and purification of PAA-SG were accomplished using the method we described for the synthesis and purification of clofibryl-*S*-acyl glutathione (Grillo and Benet, 2001). The identity of PAA-SG was confirmed by ESI/MS (Hewlett Packard HPLC 1100LC/MSD bench-top mass spectrometer) operating in positive ion mode, which provides mass spectra, *m/z*: 426 ($[MH]^+$), 351 [M + H - gly]$^+$, 308 [GSH]$^+$, 297 [M + H – pyroglytamic acid]$^+$, 194 $[C_6H_5CH_2COSCH_2\text{-}CH{=}NH_2]^+$.

9.2.4 Stability Studies

Stability studies of acyl-CoA thioesters were performed as described for similar experiments with 2-PPA-CoA (Chapter 2 and Li et al., 2002). In brief, 2,4-D-CoA (0.1 mM) or PAA-CoA (0.1 mM) was incubated at 37°C in 0.05 M potassium phosphate buffer (pH 7.4) in triplicate. Aliquots (100 µl) were taken at the indicated time points and

10 µl of the aliquot was then injected immediately into the HPLC for analysis of the products.

9.2.5 Reactions with GSH

Incubations (1 ml total volume) containing 2,4-D-CoA (0.1 mM) and GSH (1 mM) were performed at room temperature (~22°C) in 0.05 M potassium phosphate buffer (pH 7.4). Aliquots (10 µl) were taken every 20 min and analyzed by HPLC for the formation of 2,4-D-SG thioester product. ESI/MS/MS analysis was performed on a Finnigin-MAT TSQ 7000 (San Jose, CA). HPLC purified samples were introduced into the ESI source *via* a Harvard Apparatus syringe pump (Holliston, MA) at a flow rate of 0.3 ml/min. CID of the ^{12}C component of protonated 2,4-D-SG (MH^+ *m/z* 510) was performed in the collision cell. Similar incubations with GSH (1 mM) were also conducted with PAA-CoA (0.1 mM) and 2-PPA-CoA (0.1 mM).

9.2.6 Reactions with Human Serum Albumin (HSA)

Fatty acid free HSA (30 mg/ml, 0.45 mM) was incubated with [1-^{14}C]-2,4-D-CoA (0.2 mCi/mmol, 0.1 mM) or [1-^{14}C]-2,4-D (0.2 mCi/mmol, 0.1 mM, negative control) in 0.05 M potassium phosphate buffer (10 ml, pH 7.4) at 37°C in triplicate. After 0.03, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h of incubation, aliquots (1 ml) were taken and added to a solution of perchloric acid (7%, 0.5 ml), vortex mixed, and centrifuged in 15 ml Falcon polypropylene conical tubes (1500 *g*) for 10 min. The supernatants were neutralized by 1 M NaOH and injected immediately into the HPLC to determine the stability of 2,4-D-CoA in HSA solutions. Covalent binding of 2,4-D to HSA was measured by scintillation

counting of exhaustively washed protein precipitates, as described in Chapter 2. Reactions of [1,2-^{14}C]-PAA-CoA (0.2 mCi/mmol, 0.1 mM) and [1,2-^{14}C]-PAA (0.2 mCi/mmol, 0.1 mM) with HSA were done similarly.

9.2.7 HPLC Analysis

HPLC analysis was carried out on a Shimadazu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadazu Corp., Japan) with a Shimadazu SCL-10A controller and a Shimadazu SPD-10A UV-Vis detector. All reactions containing 2,4-D-CoA were analyzed on a Microsorb-MV C_8 column (150 x 4.6 mm, 5 μm, Rainin LC and Supplies, Walnut Creek, CA) at a flow rate of 1.5 ml/min. The isocratic running buffer containing 24 % acetonitrile in 0.19 M ammonium acetate buffer (pH 7.0) was used with UV detection at 226 nm. Analysis of incubations with PAA-CoA was performed by isocratic elution on a Microsorb-MV C_{18} column (150 x 4.6 mm, 5 μm, Rainin LC and Supplies, Walnut Creek, CA) at a flow rate of 1.0 ml/min. The mobile phase contained 33% methanol in 0.05 M potassium phosphate buffer (pH=5.7) and UV detection was at 226 nm. Reactions of 2-PPA-CoA with GSH at room temperature were analyzed by HPLC as reported previously (Chapter 2 and Li et al., 2002). Standard curves for quantitation were made by adding authentic standards to phosphate buffer or HSA solutions.

9.2.8 Hepatocyte Incubations

Hepatocytes were isolated from male Sprague-Dawley rats (240~260 g, B & K Universal, Livermore, CA) by collagenase perfusion of liver as described by Moldeus *et*

al. (1978). Greater than 85% viability was achieved routinely, assessed by trypan blue exclusion. Incubations of hepatocytes (4~5 x 10^6 cells/ml, final volume 30 ml) with [1-^{14}C]-2,4-D (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. After a 5-min equilibration period, reactions were started by the addition of [1-^{14}C]-2,4-D (1.5 ml of 20 mM in Krebs-Henseleit buffer, 0.2 mCi/mmol, final concentration 1 mM) and aliquots (4 ml) of the incubation mixture were taken at indicated times and quenched with 2 ml of 7% perchloric acid. Control incubations were conducted using hepatocytes denatured by heating (100°C) for 10 min. Irreversible binding of radiolabel to proteins was measured by scintillation counting of exhaustively washed hepatocyte protein precipitates as described previously (Chapter 4). The inhibitory effect of trimethylacetic acid (TMA) was studied by adding TMA (0.2 ml of 100 mM in Krebs-Henseleit buffer, pH 7.4, final concentration 2 mM) to hepatocyte suspensions (final volume 10 ml) 5 min before adding [1-^{14}C]-2,4-D (1 mM final concentration).

9.3 Results

9.3.1 Chemical Stability of Acyl-CoA Thioesters

The instability of 2,4-D-CoA thioester in phosphate buffer at 37°C and pH 7.4 is illustrated in Figure 9.2. Its disappearance rate was first-order ($t_{1/2} \cong 5.6$ h). HPLC analysis showed that the product is 2,4-D acid. Approximately 65% of 2,4-D-CoA was hydrolyzed in 8 h (Figure 9.2). Preliminary stability studies showed that 2,4-D-CoA is more stable under acidic conditions and lower temperatures. Synthetic 2,4-D-CoA

thioester was therefore stored at -80°C at pH 3. PAA-CoA, like 2-PPA-CoA (Chapter 2), is relatively stable at pH 7.4, 37°C (Figure 9.3).

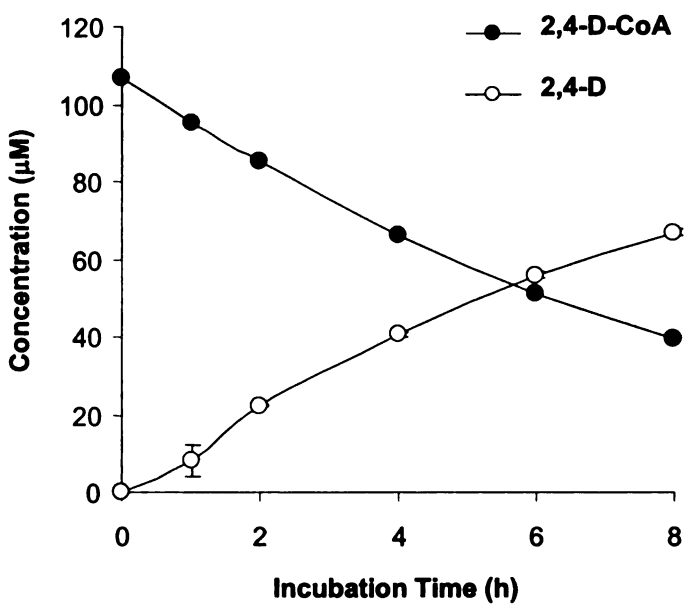
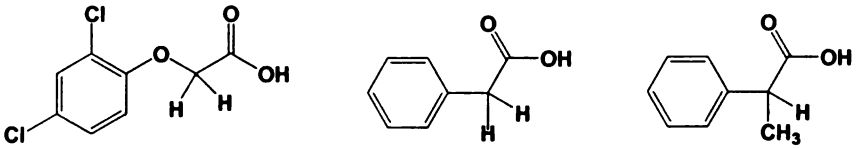


Figure 9.2 Time-course of the hydrolysis of 2,4-D-CoA thioester (0.1 mM) to 2,4-D acid in buffer (0.05 M potassium phosphate, pH 7.4, 37°C). Values are expressed as the means ± SD of triplicate incubations.

9.3.2 Reactions of Acyl-CoA Thioesters with GSH

Incubations of 2,4-D-CoA (0.1 mM) with GSH (1 mM) at room temperature and pH 7.4 led to the formation of 2,4-D-SG that eluted on HPLC with a retention time

A) Structures of 2,4-D, PAA, and 2-PPA



2,4-D PAA 2-PPA

B) Stability of Acyl-CoA

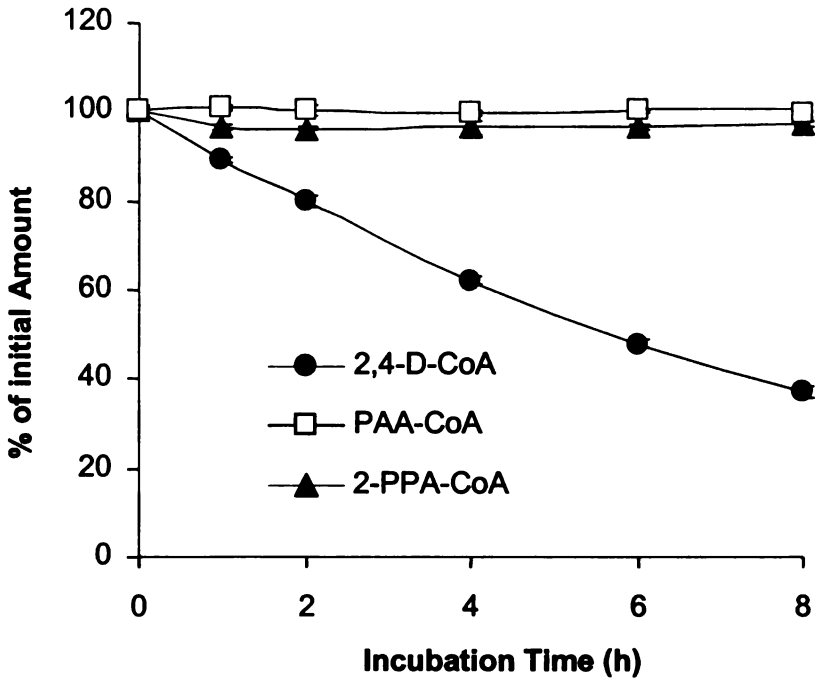


Figure 9.3 Chemical structures of 2,4-dichlorophenoxyaetic acid (2,4-D), phenylacetic acid (PAA), and 2-phenylpropionic acid (2-PPA) (A). Time-dependent degradation of 2,4-D-CoA, PAA-CoA, and 2-PPA-CoA thioesters (0.1 mM) in 0.05 M potassium phosphate (pH 7.4) at 37°C (B). Values shown represent the mean ± SD (n = 3).

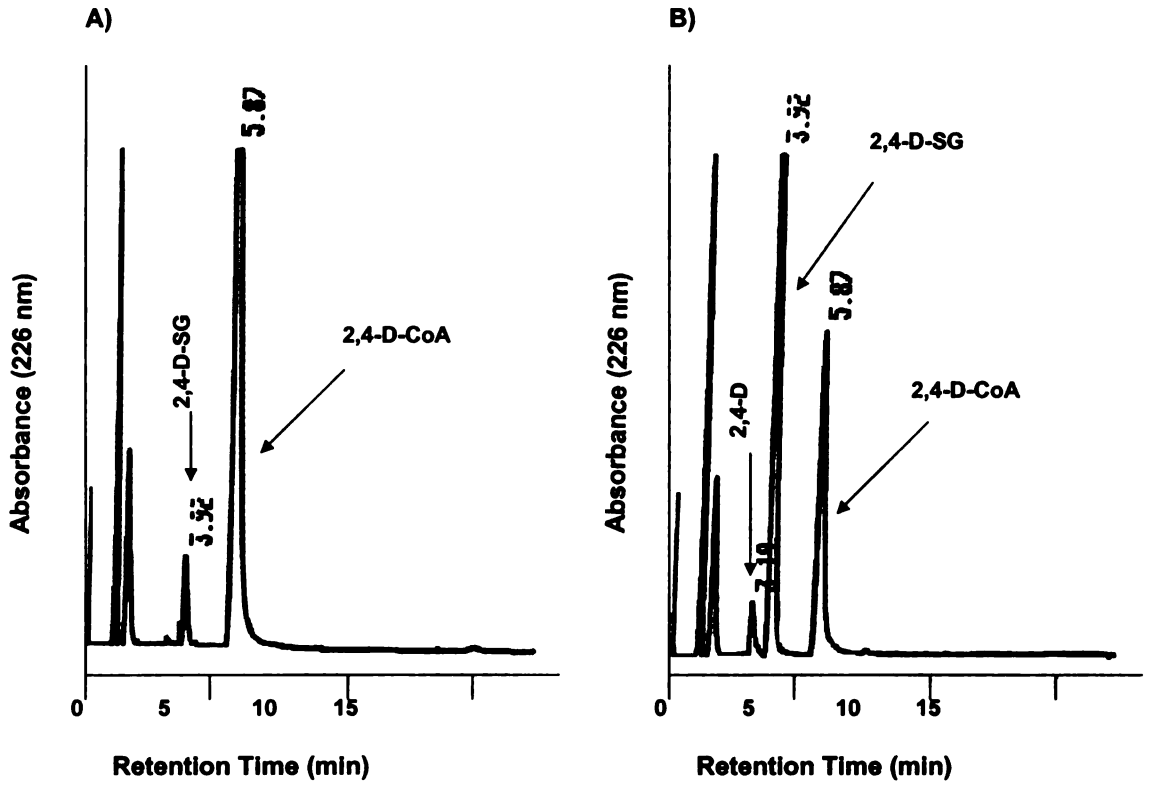


Figure 9.4 Representative reverse-phase HPLC chromatograms with UV detection (226 nm) of incubations of 2,4-D-CoA (0.1 mM, retention time 5.87 min) with GSH (1 mM) in 0.05 M potassium phosphate buffer (pH 7.4) at room temperature (22°C). Aliquots of the incubation mixture were taken and analyzed immediately by HPLC after incubation times of A) 2 min and B) 1 h. Retention times of 2,4-D and 2,4-D-SG as determined using authentic standards were 3.19 min and 3.92 min, respectively.

identical to the authentic 2,4-D-SG standard (3.92 min, Figure 9.4). HPLC analysis showed that 2-PPA-SG formation was evident after a 2-min incubation. After 1 h incubation, 65% of 2,4-D-CoA was converted to 2,4-D-SG and only 5% was hydrolyzed to 2,4-D free acid. The identity of the 2,4-D-SG reaction product was confirmed by

tandem mass spectrometric analysis (positive ion mode) of the protonated molecular ion (MH^+ *m/z* 510) and provided a product ion spectrum consistent with its structure and identical to synthetic 2,4-D-SG standard (Figure 9.5). Time-dependent studies showed that formation of 2,4-D-SG was the predominant reaction during the first 2.7 h of incubation, with an apparent 65 μM plateau being reached after 1 h (Figure 9.6). A relatively small amount of hydrolysis product (2,4-D acid) was detected, which increased gradually (4.8 μM/h) over time.

Analysis of the chemical reactivity of PAA-CoA and 2-PPA-CoA with GSH showed that *S*-acyl glutathione conjugates of PAA and 2-PPA were detectable under the same incubation conditions. Their rates of formation, however, were much slower than that for 2,4-D, i.e., 5.5 μM/h for PAA-SG and 1.4 μM/h for 2-PPA-SG (Figure 9.7).

9.3.3 Irreversible Binding Studies with HSA

Having demonstrated that 2,4-D-CoA readily acylated the GSH sulfhydryl group, we then investigated whether a similar transacylation reaction occurs with protein nucleophiles. [1-^{14}C]-2,4-D-CoA thioester (0.1 mM) was incubated with fatty acid free HSA (30 mg/ml) in potassium phosphate buffer (pH 7.4) at 37°C. Irreversible binding to HSA was evident after 0.25 h incubation, but was not detected in control incubations with [1-^{14}C]-2,4-D acid. The time-dependent irreversible binding of 2,4-D, via 2,4-D-CoA, to HSA is shown in Fig. 9.8A. Binding rapidly achieved a maximum of 440 pmol/mg HSA after 1 h and subsequently declined with incubation time (300 pmol/mg HSA at 24 h).

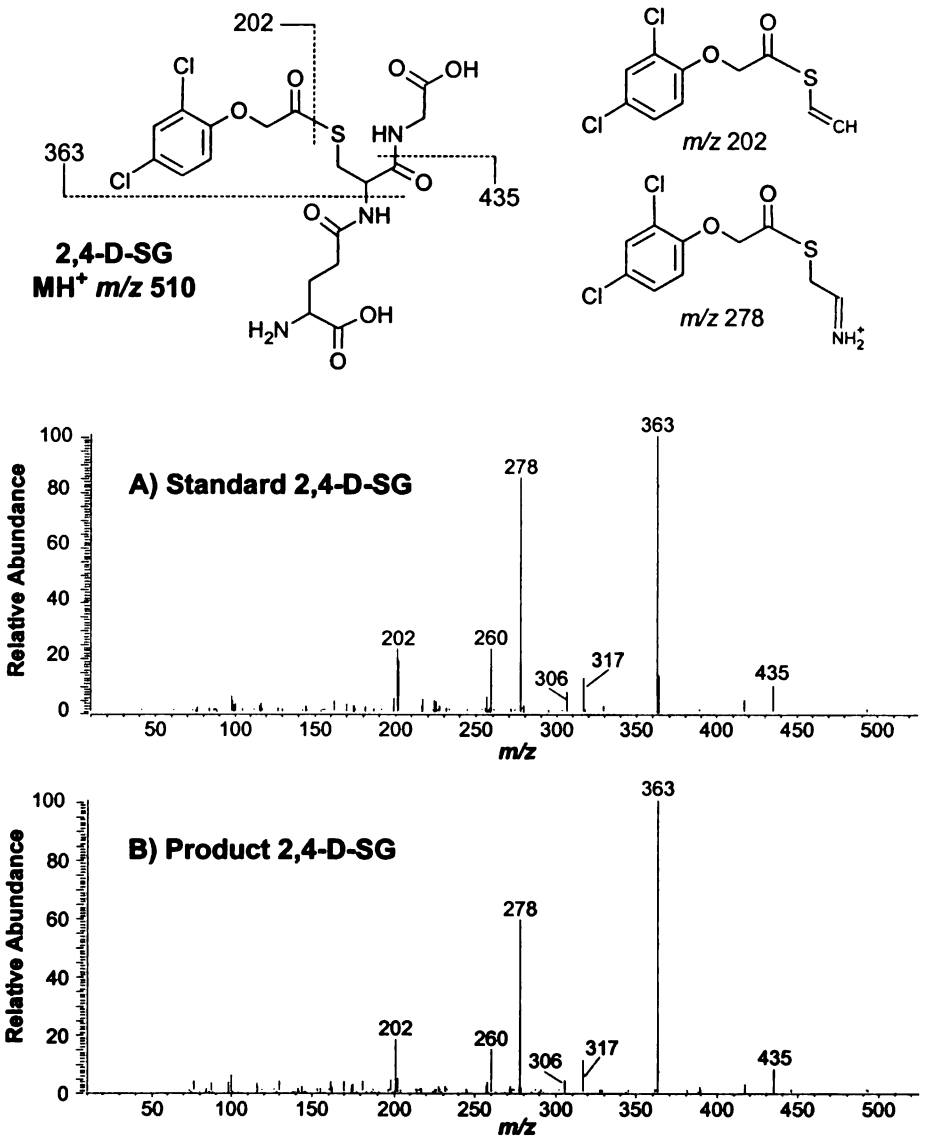


Figure 9.5 Product ion mass spectrum of A) standard 2,4-D-SG and B) 2,4-D-SG isolated from incubations of 2,4-D-CoA with GSH. Tandem mass spectra were obtained by collision induced dissociation of the protonated molecular ion MH^+ *m/z* 510.

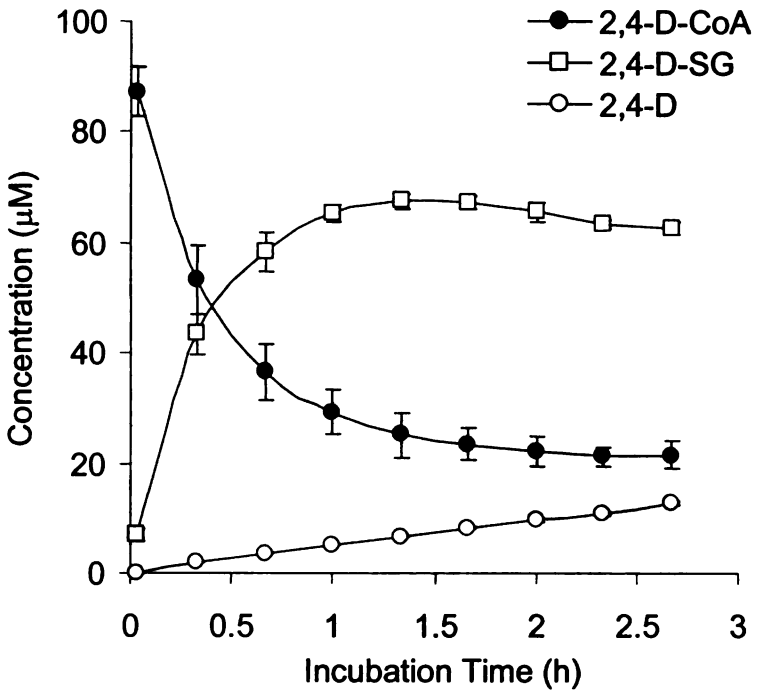


Figure 9.6 Time-dependent reaction of 2,4-D-CoA (0.1 mM) with GSH (1 mM) forming 2,4-D-SG and 2,4-D acid in 0.05 M potassium phosphate buffer (pH 7.4) at room temperature (22°C). Values represent means ± SD from triplicate incubations.

The extent of binding appears to be temporally related to the concentration of 2,4-D-CoA (*vs.* the 2,4-D acid concentration) in the incubations. HPLC analysis showed that 2,4-D-CoA is unstable in HSA solution with an apparent half-life of 0.4 h (Figure 9.8B). After 2 h incubation, 2,4-D-CoA was hydrolyzed to 2,4-D acid and no longer detectable by HPLC. Control incubations with [1-^{14}C]-2,4-D exhibited no time-dependent irreversible binding.

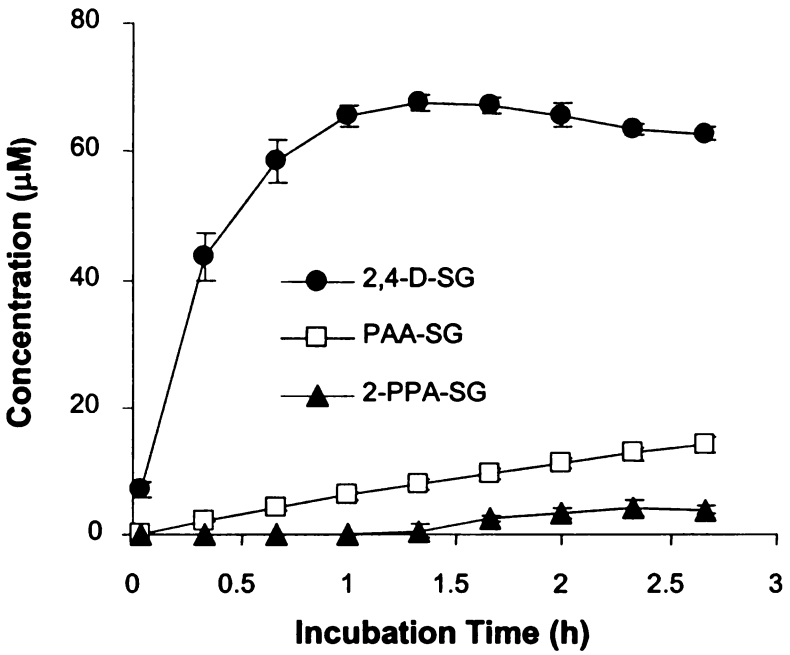


Figure 9.7 Time-dependent formation of *S*-acyl glutathiones from reactions of acyl-CoA thioesters (0.1 mM), 2,4-D-CoA, PAA-CoA, and 2-PPA-CoA, with GSH (1 mM) in phosphate buffer (pH 7.4, room temperature). Values represent means ± SD from triplicate incubations.

Irreversible binding of PAA, via PAA-CoA, to HSA increased gradually with incubation time and the maximum was not achieved at 24 h. Product formation (50 pmol/mg HSA/h, 0.0033 mole/mole HSA/h) was linear for the first 8 h. By 24 h, 630 pmol/mg (0.042 mole/mole) of protein binding was detected (Figure 9.9). HPLC analysis showed that PAA-CoA thioester was also not stable in HSA solutions, being slowly hydrolyzed to PAA acid. The half-life of PAA-CoA in HSA solution was approximately 24.3 h (data not shown).

As reported previously (Chapter 3), irreversible binding of 2-PPA, via 2-PPA-CoA, to HSA (100 pmol/mg HSA/h; 0.0066 mole/mole HSA/h) approached linearity during the first 8 h incubation. After a 24-h incubation, binding density was 0.064 mole/mole HSA (970 pmol/mg HSA). The degradation of 2-PPA-CoA thioester to 2-PPA acid in HSA solution followed first-order kinetics with an apparent half life of 13.4 h (Chapter 3).

9.3.4 Irreversible Binding Studies in Freshly Isolated Hepatocytes

Exposure of hepatocytes to [1-^{14}C]-2,4-D (1 mM) yielded time-dependent irreversible binding, which was significantly greater than that found with heat denatured hepatocytes, indicating that reactive metabolites are formed in hepatocytes (Figure 9.10A). The reason for the non-zero values at time 0 h for experimental and control studies is not known.

To further examine whether 2,4-D-CoA can bind irreversibly to rat hepatic proteins, inhibition studies were run with [1-^{14}C]-2,4-D (1 mM) in the presence of TMA (2 mM), which competitively inhibits acyl-CoA formation (Fig. 9.10B). Covalent binding of 2,4-D to hepatic proteins decreased by 50% in hepatocytes treated with TMA (2 mM), compared to controls.

9.4 Discussion

The extensive use of 2,4-D as a defoliant has stimulated extensive research on its toxicity to animals and humans (Stevens and Sumer, 1991). Liver toxicity has been

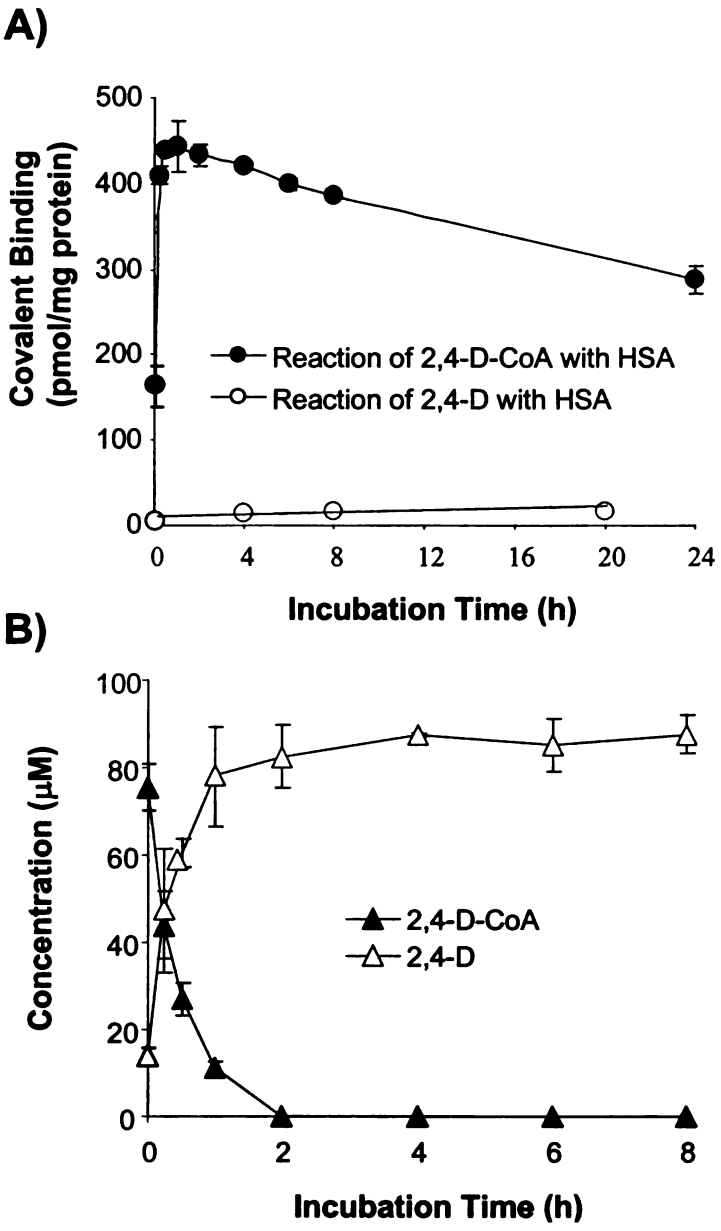


Figure 9.8 Time-dependent covalent binding (A) and chemical stability (B) of [1-^{14}C]-2,4-D-CoA (0.1 mM) in 0.05 M potassium phosphate buffer containing HSA (30 mg/ml, 0.45 mM) at pH 7.4 and 37°C. Incubation with 0.1 mM [1-^{14}C]-2,4-D-CoA (o) under the same conditions served as a negative control (A). Values represent means ± SD from triplicate incubations.

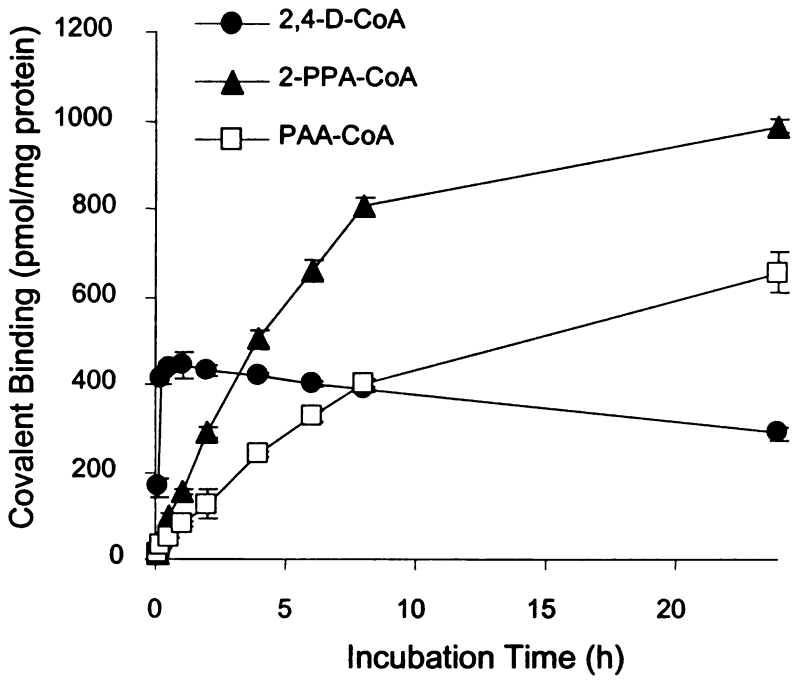


Figure 9.9 Time-dependent covalent binding of acyl-CoA thioesters (0.1 mM), [1-^{14}C]-2,4-D-CoA, [1,2-^{14}C]-PAA-CoA, and [1-^{14}C]-2-PPA –CoA, to HSA (30 mg/ml, 0.45 mM) in 0.05 M potassium phosphate buffer (pH 7.4) at 37°C. Values represent means ± SD from triplicate incubations. 2-PPA-CoA data previously reported in Chapter 3.

demonstrated, but little is known about the underlying mechanisms. Reactive metabolites of xenobiotics can sometimes cause toxicity by binding covalently to proteins (Pumford and Halmes, 1997). Covalent binding of 2,4-D to hepatic proteins has been demonstrated in vivo in rat and chick (Evangelista de Duffard et al., 1993; Di Paolo et al., 2001). The reactive metabolites of 2,4-D that mediate such covalent binding, however, have not been identified. In the present study, we show that 2,4-D- CoA thioester is a reactive metabolite of 2,4-D that readily undergoes transacylation reactions with GSH, resulting

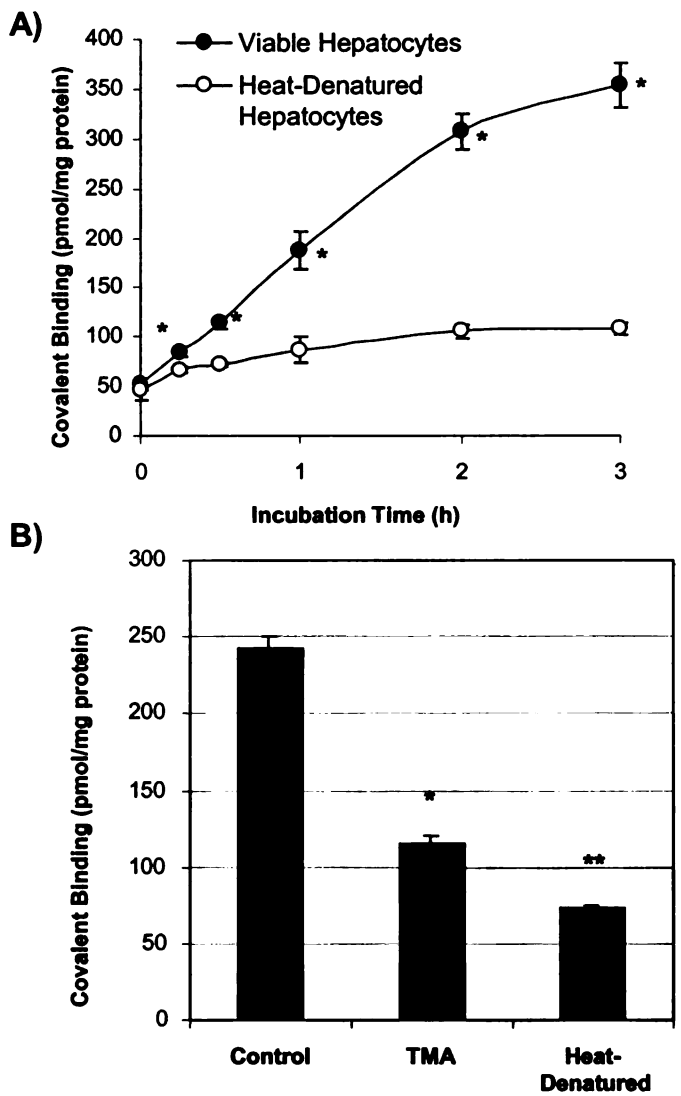


Figure 9.10 Irreversible binding of 2,4-D to hepatic proteins in freshly isolated hepatocytes. A) Time-dependent irreversible binding from incubations of [1-^{14}C]-2,4-D (1 mM) with freshly isolated rat hepatocytes (4 million cells per ml) and heated-denatured hepatocytes (negative control). B) Inhibitory effects of trimethylacetic acid (TMA, 2 mM) on irreversible binding of 2,4-D to hepatocyte proteins after 3 h of incubations. Values represent means ± SD from triplicate incubations. *, $p < 0.01$; **, $p < 0.001$, versus control.

in the formation of a 2,4-D-SG thioester conjugate, and with nucleophilic residues on proteins resulting in 2,4-D-protein adduct formation.

Unlike other xenobiotic acyl-CoA thioesters studied to date, the 2,4-D-CoA derivative is chemically unstable at physiological pH and temperature, with an apparent half-life of 5.6 h. After 8 h incubation, approximately 60% of 2,4-D-CoA was hydrolyzed to 2,4-D acid (Figure 9.2). In contrast, hydrolysis of PAA-CoA or 2-PPA-CoA was not detectable during 8 h incubation (Figure 9.3). In vitro studies showed that 2-PPA-CoA thioester is very stable, with an apparent half-life of 12 days at pH 7.4 and 37°C (Chapter 2 and Li et al., 2002). Similar chemical stability was observed for clofibryl-*S*-acyl-CoA under the same conditions (Grillo and Benet, 2002). The unstable nature of 2,4-D-CoA thioester in buffer suggested that 2,4-D-CoA might be more electrophilic in transacylation-type reactions than other xenobiotic acyl-CoA thioesters studied. This suggestion was supported by our previous observation on the transacylation of *S*-acyl-CoA thioesters with GSH.

Glutathione, a tripeptide with a nucleophilic cysteinyl thiol, reacts with electrophiles and is a useful substrate for characterizing the electrophilicity of xenobiotic metabolites. At room temperature and pH 7.4, 2,4-D-CoA (0.1 mM) reacts with the nucleophilic cysteinyl thiol of excess GSH (1 mM) to form 2,4-D-SG. Formation of 2,4-D-SG is time-dependent and reaches an apparent plateau 65 μM after 1 h of incubation (Figure 9.6). Under identical conditions, the reactions of PAA-CoA and 2-PPA-CoA with GSH were much slower (Figure 9.7).

The reactivities of the three acyl-CoA thioesters towards water and glutathione appear to be highly sensitive to the substituent at the acyl α-carbon. 2,4-D-CoA thioester, which was most reactive, contains an electron-withdrawing oxygen atom adjacent to the α-carbon of the carboxyl group and two electron-withdrawing substituents in the benzene ring (Figure 9.3A). In contrast, the corresponding carbon in PAA is unsubstituted and connected directly to an electron-rich benzene ring (Figure 9.3A). 2-PPA, with one α-carbon substituent, was the least reactive. Similar structure-activity relationships have been demonstrated for acyl glucuronides and *S*-acyl glutathione (Benet et al., 1993; Grillo and Benet, 2002). Accumulated data from several studies of acyl glucuronides suggest that the degree of substitution at the α-carbon can be used to predict the likelihood of their hydrolysis, acyl migration and covalent binding to protein in vitro and in vivo (Benet et al., 1993). Grillo and Benet (2002) have recently shown a similar relationship for transacylation reactions of *S*-acyl glutathione derivatives with N-acetylcysteine (NAC). The rank order of reactivity of the *S*-acyl glutathione derivatives with NAC was phenoxyacetyl- > arylacetyl- > 2-phenylpropionyl- = α,α-dimethyl-phenoxyacetyl- > α,α-dimethyl-substituted *S*-acyl glutathione (Grillo and Benet, 2002), which is consistent with our transacylation reactions of acyl-CoA thioesters.

Having demonstrated that 2,4-D-CoA thioester is readily transacylated by GSH, we investigated whether similar transacylation occurs with HSA. Incubating [1-^{14}C]-2,4-D-CoA thioester (0.1 mM) with fatty acid free HSA (30 mg/ml, 0.45 mM) resulted in time-dependent irreversible binding of 2,4-D to HSA, which did not occur in control incubations with [1-^{14}C]-2,4-D acid. Irreversible binding peaked at 440 pmol/mg HSA (0.029 mole/mole) after 1 h of incubation and then declined slowly, presumably because

of hydrolysis of the drug-protein products (Figure 9.8A). Consistent with previous studies with 2-PPA-CoA (Chapter 3) hydrolysis of 2,4-D-CoA to 2,4-D acid was markedly accelerated by HSA (Figure 9.8B). The apparent half-lives of 2,4-D-CoA in the presence and absence of HSA were 0.4 h and 5.6 h, respectively, suggesting that HSA has esterase activity towards the thioester.

The rank order of the initial rate of irreversible binding of the three studied acyl-CoAs to HSA was 2,4-D-CoA > 2-PPA-CoA > PAA-CoA, which did not correlate with the carboxyl α substitution (Figure 9.9). However, the rank order of the rate of irreversible binding did correlate with the stability of the thioesters in HSA solution, where the apparent half-lives of 2,4-D-CoA, 2-PPA-CoA and PAA-CoA were 0.4 h, 13.4 h, and 24.3 h, respectively. Thus, the degradation rate appears to be the predictor, as found by Benet et al. (1993) for some acyl glucuronides.

Irrversible binding of acyl-CoA thioesters to proteins is probably more complex than direct transacylation by nucleophiles such as GSH. In addition to their intrinsic reactivity, the reversible binding of the thioesters to the protein may also be important. Reversible binding is generally favored by high lipophilicity. The rank order of lipophilicity of the three carboxylic acids is 2,4-D > 2-PPA > PAA, which correlates well with the irreversible binding and stability of their acyl-CoA thioesters in HSA solution. The stability of acyl-CoA thioesters in HSA solution, rather than in buffer, may be a good predictor for their in vitro irreversible binding to HSA, since both processes are affected by both the chemical structure of the ester and its reversible interaction with the protein.

Our in vitro studies show that 2,4-D-CoA thioester reacts irreversibly with large molar excesses of GSH and HSA. To examine if 2,4-D-CoA binds irreversibly to hepatic

proteins, we incubated freshly-isolated hepatocytes with [1-^{14}C]-2,4-D. Time-dependent irreversible binding to hepatic protein was detected in viable, but not in heat-denatured hepatocytes (Figure 9.10A). Pretreatment with TMA (2 mM), an inhibitor of acyl-CoA formation, decreased irreversibly binding by 50% (Figure 9.10B). These observations suggest that 2,4-D-CoA formation is responsible for the irreversible binding.

This is the first study of the stability and reactivity of the CoA metabolite of the herbicide 2,4-D. My in vitro data suggest that this metabolite can bind irreversibly to HSA and hepatic proteins. If such a metabolite is formed in humans, it could contribute to the hepatotoxicity of 2,4-D (Leonard et al., 1997).

Chapter 10

Conclusions and Perspectives

10.1 Overview

Acyl glucuronide metabolites of acidic drugs are well-known reactive metabolites that can bind covalently to protein and may potentially contribute to the associated heptotoxic effects. During the last two decades, great attention has been focused on the acyl glucuronide metabolites of xenobiotic carboxylic acids. Covalent adduct formation of acidic drugs is generally attributed to acyl glucuronides. We believe that an alternative metabolic activation pathway of xenobiotic carboxylic acids, namely acyl-CoA formation, may also contribute to the covalent binding to hepatic protein and thus potentially mediate some of toxic side-effects associated with these drugs. In this thesis, I used a model carboxylic acid, 2-PPA, to study the chemical reactivity of xenobiotic acyl-CoA thioesters and compare it with that of the corresponding acyl glucuronides. The results provide strong evidence that xenobiotic acyl-CoA derivatives are reactive acylating reagents that, in addition to acyl glucuronides, contribute to the covalent binding of acidic drugs to proteins and might be responsible for their idiosyncratic toxicity.

10.2 Characterization of the Chemical Reactivity of 2-PPA-CoA Towards Protein Nucleophiles

The chemical reactivity of 2-PPA-CoA thioester towards protein nucleophiles was examined in vitro using human serum albumin (HSA) and rat liver homogenate (Chapter 3). Incubations of 2-PPA-CoA with HSA in phosphate buffer showed that 2-PPA-CoA bound irreversibly to HSA over time with 970 pmol/mg HSA being reached after 24 h. I also demonstrated that covalent binding of 2-PPA to rat liver homogenate was dependent on 2-PPA-CoA formation. When (*RS*)-[1-^{14}C]-2-PPA was incubated with rat liver homogenate and cofactors for acyl-CoA formation (CoA, ATP and Mg^{2+}), both 2-PPA-CoA formation and covalent binding were detected. In the absence of ATP, no 2-PPA-CoA was formed and little covalent binding was detected. Inhibition of 2-PPA-CoA formation significantly decreased the irreversible binding. Thus the 2-PPA-CoA derivative could be an important reactive metabolite, causing covalent adduct formation in the liver.

10.3 Comparison of the Chemical Reactivity of 2-PPA-CoA Thioester and Acyl Glucuronide

10.3.1 Reaction with GSH

Glutathione (GSH), a cytoprotective tripeptide with a nucleophilic cysteinyl thiol, reacts directly with electrophiles and is a useful model for comparing the electrophilicity of reactive metabolites of xenobiotics. Presumably, the greater the reactivity of the acylating metabolite with GSH, the greater the probability that the metabolite will acylate

protein nucleophiles, and the greater the risk of toxicity. Therefore, I characterized and compared the chemical reactivities of the acyl glucuronide and acyl-CoA thioester derivatives of 2-PPA with GSH in vitro in buffer (Chapter 2 and Li et al., 2002) and in vivo in rats (Chapter 8).

In Vitro Studies. Incubations of synthetic 2-PPA-CoA and biosynthetic 2-PPA-1-*O*-acyl glucuronide (2-PPA-1-*O*-G) with GSH were done in phosphate buffer (Chapter 2 and Li et al., 2002). HPLC analysis of the products from both reactions showed the presence of 2-PPA-SG, which was confirmed by coelution with authentic 2-PPA-SG as well as by LC/MS. The formation of 2-PPA-SG was time- and concentration-dependent with a formation rate constant of $(1.9 \pm 0.2) \times 10^{-2}$ $M^{-1} \cdot s^{-1}$ from reactions of GSH with 2-PPA-CoA, and $(2.7 \pm 0.4) \times 10^{-4}$ $M^{-1} \cdot s^{-1}$ from reactions of GSH with 2-PPA-1-*O*-G. The reactivity of GSH with 2-PPA-CoA appears to be 70 times greater than with 2-PPA-1-*O*-G, but there may have been an artifact caused by acyl migration of the glucuronide to less reactive isomers and hydrolysis. The results support the hypothesis that xenobiotic acyl-CoA thioesters are reactive acylating species that may contribute to xenobiotic acid-protein adduct formation in vivo.

In Vivo Studies. The (*R*)- and (*S*)-enantiomers of 2-PPA are metabolized in vivo to chemically reactive acyl glucuronide and acyl-CoA thioester derivatives, which both transacylate GSH to form 2-PPA-SG in buffer, as shown in Chapter 2 (Li et al., 2002). The conversion of 2-PPA to the acyl glucuronide is enantioselective for the (*S*)-enantiomer ($R/S = 0.6$), whereas acyl-CoA formation of 2-PPA is enantioselective for the (*R*)-enantiomer ($R/S = 7.5$), in vivo in rat liver (Chapter 8). I used these enantioselective differences in metabolism to determine which reactive metabolite

acylates GSH in vivo (Chapter 8). Studies were performed to examine the glutathione-derived products excreted in urine and bile after dosing rats separately with (*R*)- or (*S*)-2-PPA (130 mg/kg). LC/MS/MS analyses showed the presence of 2-PPA-SG thioester in the bile and the rearranged degradation product 2-PPA-*N*-acyl cysteine (2-PPA-NC) conjugate in the urine from rats dosed with (*R*)- or (*S*)-2-PPA. The total formation of 2-PPA-SG in vivo in rats, as indicated by the total amount of 2-PPA-SG-derived products excreted in urine and bile, was enantioselective for the (*R*)-isomer (*R/S* = 3.3), which correlated more closely with the enantioselectivity of acyl-CoA formation than that of acyl glucuronidation of (*R*)- and (*S*)-2-PPA enantiomers. Results from these studies strongly suggest that metabolic activation of 2-PPA by acyl-CoA formation provides more reactive acylating species in the liver than acyl glucuronidation. We propose that acyl-CoA thioester derivatives of carboxylic acid-containing drugs, besides acylating glutathione, contribute to the acylation of protein nucleophiles in vivo and possibly cause some of the toxic side effects associated with the use of these drugs.

10.3.2 Irreversible Binding of 2-PPA to Hepatic Protein: Hepatocyte Studies

A series of studies was conducted to investigate the potential of (*R*)- and (*S*)-2-PPA to undergo enantioselective covalent binding to protein in freshly isolated rat hepatocytes and to determine if such covalent binding is dependent on acyl glucuronidation or acyl-CoA formation of 2-PPA (Chapter 4). 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 (*RS*)- [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) to inhibit 2-PPA-CoA formation and 2-PPA acyl glucuronidation, respectively. Covalent binding of 2-PPA to hepatocyte protein decreased 53% in cells treated with trimethylacetic acid, and the concentration of 2-PPA-CoA decreased 66%. Conversely, (-)-borneol, which completely inhibited 2-PPA acyl glucuronidation, decreased covalent binding by only 18.7%. These results are consistent with the hypothesis that metabolism of 2-PPA by acyl-CoA formation generates a reactive thioester that forms more protein adducts than the acyl glucuronide.

10.3.3 Covalent Binding of 2-PPA to Hepatic Protein: In Vivo Studies

The relative importance of the two metabolic activation pathways of 2-PPA, namely acyl-CoA formation and acyl glucuronidation, was also examined in vivo in rats (Chapter 5). Male Sprague-Dawley rats were pretreated with and without (-)-borneol (320 mg/kg, i.p., 30 min), an inhibitor of acyl glucuronidation, or trimethylacetic acid (TMA, 500 mg/kg, i.p., 10 min), an inhibitor of acyl-CoA formation, prior to receiving (*R,S*)-2-PPA (130 mg/kg). Pretreatment with TMA decreased irreversible binding of 2-PPA to liver proteins 49%, while increasing the hepatic concentration of 2-PPA-CoA by 70%. Conversely, 95% inhibition of acyl glucuronidation by (-)-borneol led to a 22% decrease in irreversible binding to protein. These results strongly suggest that metabolic activation by CoA formation contributes more to protein adduct formation in the liver *in vivo* than activation by acyl glucuronidation for 2-PPA.

10.4 Regulation of 2-PPA-CoA Formation and Its Effect on 2-PPA Adduct Formation

10.4.1 Studies with Clofibric Acids

Clofibric acid (CA), a hypolipidemic agent, is a potent peroxisomal proliferator in rats (Hawkins et al., 1987). It induces several hepatic enzymes associated with fatty acid metabolism, including acyl-CoA synthetases, the enzymes that catalyze the formation of acyl-CoA thioesters (Alegret et al., 1994, Schoonjans et al., 1993). A significant increase in hepatic free CoA, an essential cofactor for acyl-CoA formation, also occurs in CA-treated rats (Horie et al., 1986). Therefore, we hypothesized that CA treatment might increase covalent binding of 2-PPA by increasing the exposure of liver proteins to 2-PPA-CoA thioester (Chapter 6). My studies showed that exposure of 2-PPA-CoA in rat liver during the 2 h period increased significantly (2.9 fold, $p < 0.01$) on CA treatment, as did 2-PPA-CoA synthetase activity in liver homogenate (357 ± 50 *vs.* 45.2 ± 13.7 pmol 2-PPA-CoA formed/min/mg protein relative to control, $p < 0.001$). Conversely, CA treatment decreased 2-PPA acyl glucuronidation 21%. Irreversible binding of 2-PPA to rat liver protein was increased significantly (25%, $p < 0.05$) in CA-treated rats. These results support the hypothesis that CA-treatment increases irreversible binding and may, therefore, augment the risk of the idiosyncratic toxicity associated with the use of 2-arylpropionic acids.

10.4.2 Diabetes Studies

Experimental models of diabetes mellitus are characterized by a number of features that could change the metabolic activation of 2-arylpropionic acids. For example in streptozotocin-treated rats, a model of insulin-dependent diabetes mellitus (type 1), there are marked increases in hepatocellular CoASH concentration (Horie et al., 1986) and the activity of long-chain acyl-CoA synthetase, the enzyme that catalyzes acyl-CoA thioester formation (Asayama et al., 1999). Diabetes also changes the glucuronidation of many drugs in rats (Eacho et al., 1981; Price and Jollow, 1982; Grant and Duthie, 1987). Therefore, I examined the effects of streptozotocin-induced diabetes on metabolic activation of 2-PPA in freshly isolated hepatocytes (Chapter 7). Diabetic rats exhibited significant induction of 2-PPA-CoA thioester formation in hepatocytes. The exposure of 2-PPA-CoA to rat hepatic proteins over a 3 h incubation increased up to 5-fold ($p < 0.001$) in the diabetic rats. There was also a 2-fold increase in 2-PPA acyl glucuronidation ($p < 0.05$). As expected from the marked increase in 2-PPA-CoA formation and acyl glucuronidation, a significant increase in covalent binding up, to 2.54 fold ($p < 0.001$), was observed in diabetic rats 3 h after hepatocyte incubations. These results indicate that diabetes might increase the risk of idiosyncratic toxicity from 2-arylpropionic acid drugs.

10.5 Characterization of the Chemical Reactivity of 2,4-D-CoA

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used broadleaf herbicide that has been associated with acute liver toxicity in humans or animals. Metabolic activation of 2,4-D by acyl-CoA formation and subsequent irreversible binding to protein is another

possible cause of 2,4-D-induced hepatotoxicity. This led us to study the chemical reactivity of 2,4-D-S-acyl CoA (2,4-D-CoA) in vitro. We found that 2,4-D-CoA: (a) undergoes hydrolysis in buffer (pH 7.4), with an apparent half-life of 5.6 h; (b) transacylates the cysteine sulfhydryl of glutathione, resulting in the formation of 2,4-D-*S*-acyl glutathione (2,4-D-SG) thioester with maximum yield of 65 μM after 1 h incubation; and (c) reacts with HSA, with maximum conjugate formation of 440 pmol/mg HSA within 1 h. In addition, incubation of [1-^{14}C]-2,4-D (1 mM) with rat hepatocytes showed time-dependent irreversible binding of 2,4-D to hepatocyte protein. Inhibition of acyl-CoA formation by trimethylacetic acid (TMA, 2 mM) decreased the amount of irreversible binding in rat hepatocytes by 50%. These results indicate that 2,4-D-CoA thioester is a reactive metabolite of 2,4-D that may contribute to 2,4-D-protein adduct formation *in vivo* and to 2,4-D's hepatotoxicity.

10.6 Concluding Remarks

Acyl glucuronidation and acyl-CoA formation are competitive processes for carboxylic acids. Acyl glucuronidation has been observed in a broad range of xenobiotic carboxylic acids, and quantitively, is the most important route of metabolism of acidic drugs. In contrast, studies of xenobiotic acyl-CoA formation have been restricted to 2-arylpropionic, aryloxyacetic and aromatic carboxylic acids. There is limited information concerning whether other carboxylic acids, including arylacetic acids (e.g., diclofenac and indomethacin), diuretic agents (e.g., furosemide and ethacrynic acid), and HMG-CoA redutase inhibitors (statins), form acyl-CoA thioesters in vivo.

Here, I show that metabolic activation of 2-PPA by acyl-CoA formation contributes more to irreversible protein binding in the liver than acyl glucuronidation in rats. However, the results cannot be extrapolated quantitatively to other species and other carboxylic acids, since the ratios of the two pathways may vary, with one metabolic pathway contributing more to adduct formation than the other, depending on xenobiotic substrate and animal species. However, both pathways must be considered in studies of the covalent binding of xenobiotic and endogenous carboxylic acids to proteins.

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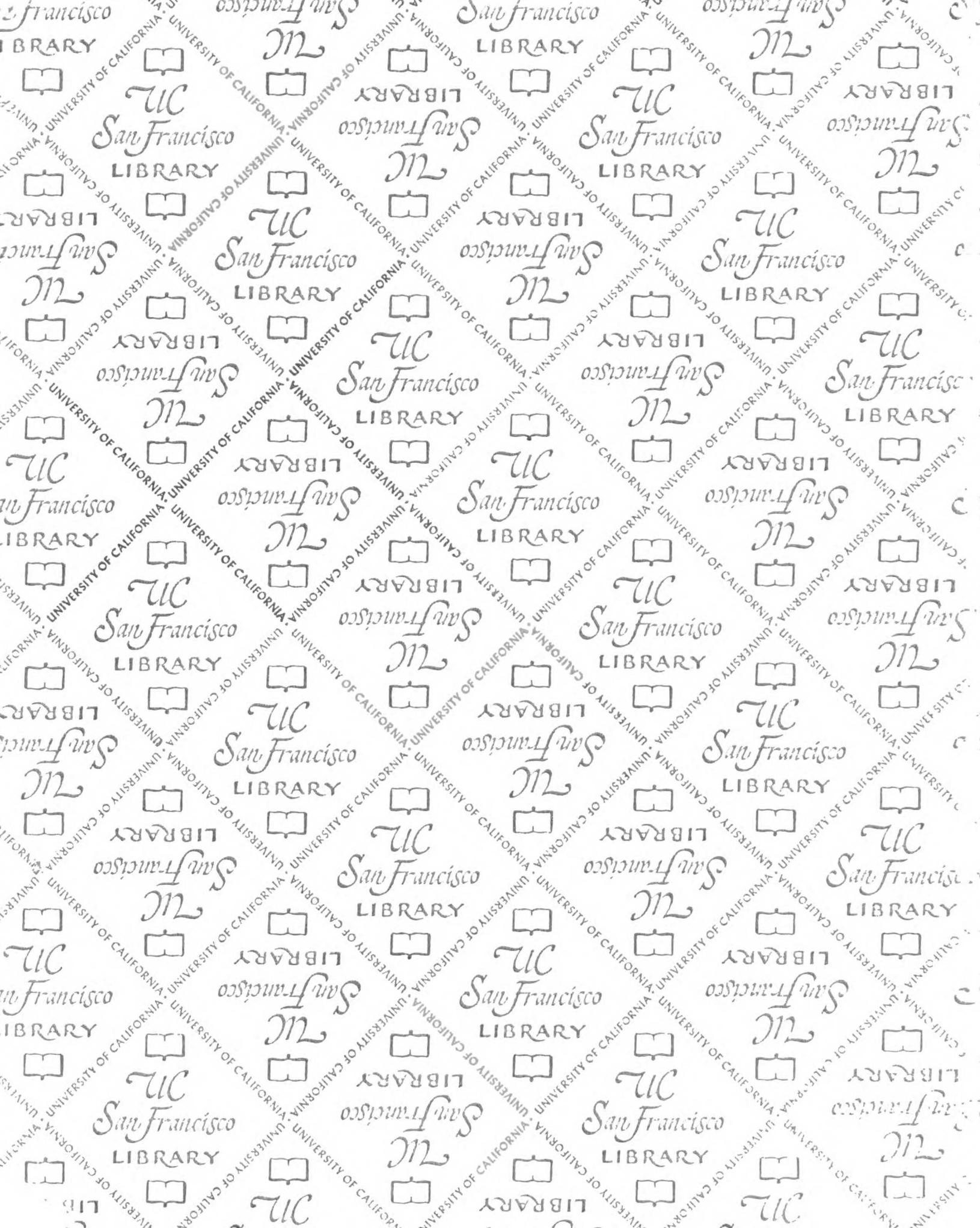
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