STUDIES ON THE REACTIVITY OF CLOFIBRYL-S-ACYL-CoA THIOESTER WITH GLUTATHIONE IN VITRO

MARK P. GRILLO and LESLIE Z. BENET

Department of Biopharmaceutical Sciences, School of Pharmacy, University of California, San Francisco, California

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ABSTRACT:

Clofibric acid (p-chlorophenoxyisobutyric acid) is metabolized in vivo to a thioester-linked glutathione conjugate, S-(p-chlorophenoxyisobutyryl)glutathione (CA-SG). The formation of this metabolite is presumed to occur via transacylation reactions between glutathione (GSH) and reactive acyl-linked metabolite(s) of the drug. The present study examines the chemical reactivity of clofibryl-S-acyl-CoA (CA-SCoA), an acyl-CoA thioester intermediary metabolite of clofibric acid, with GSH to form the CA-SG in vitro. Incubations of CA-SCoA (1 mM) with GSH (5 mM) were carried out at pH 7.5 and 37°C, with analysis of the formed reaction products by isocratic reverse-phase high-performance liquid chromatography (HPLC). Results showed a time-dependent and linear formation of CA-SG up to 4 h (50 μM CA-SG formed/h), and after a 1-day incubation, the reaction mixture contained 0.7 mM CA-SG. The identity of CA-SG was confirmed by analysis of HPLC-purified material by tandem mass spectrometry. The rate of CA-SG formation was found to be increased 3-fold in incubations containing rat liver glutathione S-transferases (4 mg/ml). Analysis of the chemical stability of CA-SCoA in buffer at 37°C and varying pH showed the derivative to be stable under mildly acidic and basic aqueous conditions but to hydrolyze at pH values greater than 10 after a 1-day incubation (t1/2 = ~1 day at pH 10.5). Results from these studies show that CA-SG is a reactive thioester derivative of clofibric acid and is able to acylate GSH and other thiol-containing nucleophiles in vitro and, therefore, may be able to acylate protein thiols in vivo, which could contribute to the toxic side effects of the drug.

Clofibric acid (CA), a carboxylic acid-containing drug and the pharmacologically active form of the hypolipidemic agent ethyl clofibrate, has been shown to bind covalently to proteins in treated patients (Sallustio et al., 1991). The formation of clofibrate-protein conjugates in vivo has been proposed to mediate the hepatotoxic effects associated with the use of CA (Faed, 1984; Sallustio et al., 1991; Boelsterli et al., 1995). Increasing evidence suggests that many carboxylic acid-containing drugs are metabolized to reactive intermediates that can form irreversible adducts with proteins (Spahn-Langguth and Benet, 1992; Bailey and Dickinson, 1991; Boelsterli et al., 1995). Such drug-protein adducts have been proposed to act as immunogens, leading to immunotoxic reactions (Spahn-Langguth and Benet, 1992; Benet et al., 1993). In addition to irreversible protein binding, CA becomes metabolized in vivo to a thioester-linked glutathione conjugate, clofibryl-S-acyl-glutathione (CA-SG; Fig. 1) (Stogniew and Fenselau, 1982; Shore et al., 1995). The covalent binding to protein and the formation of CA-SG have been proposed to occur via transacylation reactions of reactive acyl-linked metabolite(s) of CA with protein nucleophiles and GSH, respectively (Stogniew and Fenselau, 1982; Shore et al., 1995). CA is metabolized to two prospective reactive acyl-linked metabolites (Fig. 1), 1-O-acyl-clofibryl glucuronide (1-O-CAG) (Caldwell et al., 1979; Emudianughe et al., 1983) and clofibryl-S-acyl-CoA (CA-SCoA) (Bronfman et al., 1986; Lygre et al., 1986). 1-O-CAG is a major urinary metabolite of CA in humans and has been the reactive metabolite of interest in studies on the covalent binding of CA to protein nucleophiles. In vitro studies with 1-O-CAG have indicated that this metabolite is unstable due to the electrophilic nature of the carbonyl-carbon of the 1-O-acyl-linkage (van Breeman and Fenselau, 1985). The reactive acyl-linkage of 1-O-CAG undergoes hydrolysis and also rearranges, via intramolecular acyl migration reactions, with adjacent hydroxyl moieties on the glucuronidic acid ring to form 2-O-, 3-O-, and 4-O-CAG isomers (Grubb et al., 1993). In addition, the acyl-linkage reacts with nucleophilic thiols, including ethanethiol and the free cysteine sulfhydryl group of serum albumin (Stogniew and Fenselau, 1982), and GSH (Shore et al., 1995) in transacylation reactions in vitro.

Thioester-linked acyl-CoA derivatives also possess an electrophilic carbonyl-carbon (Huxtable, 1986; Brass, 1994). The activation of endogenous fatty acids to their corresponding high-energy acyl-CoA thioester derivatives is a required enzymatic step before the use of
mediated acylation of GSH provides a second mechanism, in vitro to form CA-SG. Formation of CA-SG by CA-SCoA has been shown to react spontaneously with cysteine-containing proteins, studies on the chemical reactivity of xenobiotic acyl-CoA derivatives have rarely been performed. To our knowledge, such studies include in vitro experiments on the nonenzymatic acylation of glycine by salicyl-CoA to form the glycine amide salicylate (Tisher and Goldman, 1970). It was shown that the free amine of glycine, even under conditions in which the amine is protonated (pH 7.5), was acylated by salicyl-CoA to form salicyluric acid.

Although xenobiotic acyl-CoA derivatives of acidic drugs have been considered as metabolic activation products that could acylate proteins, studies on the chemical reactivity of xenobiotic acyl-CoA derivatives have rarely been performed. To our knowledge, such studies include in vitro experiments on the nonenzymatic acylation of glycine by salicyl-CoA to form the glycine amide salicylate (Tisher and Goldman, 1970). It was shown that the free amine of glycine, even under conditions in which the amine is protonated (pH 7.5), was acylated by salicyl-CoA to form salicyluric acid. A number of studies have been conducted to characterize the nonenzymatic acylation of protein sulfhydryls by endogenous acyl-CoA derivatives in vitro (Bharadwaj and Bizzozero, 1995; Yamashita et al., 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. These findings on the reactivity of endogenous acyl-CoA derivatives with protein thiol s in vitro, we propose, are directly related to, and provide an insight into, similar reactions that may be occurring in vivo with potentially reactive xenobiotic acyl-CoA thioester derivatives formed during the metabolism of acidic drugs (Hertz and Bar-Tana, 1988; Bronfman et al., 1992; Sallustio et al., 2000). To gain a better understanding of the reactivity of xenobiotic acyl-CoA derivatives with nucleophiles, the objective of the present study was to investigate the chemical reactivity of CA-SCoA with GSH in vitro to form CA-SG. Formation of CA-SG by CA-SCoA-mediated acylation of GSH provides a second mechanism, in addition to S-acylation by 1-O-CAG, which can contribute to the formation of CA-SG in vivo (Fig. 1). We hypothesize that the acylation of GSH, as a model thiol, in vitro will provide insight into understanding the chemical reactivity of xenobiotic acyl-CoA derivatives with biological nucleophiles, such as proteins, in vivo.

### Experimental Procedures

**Materials.** CA, GSH, EDTA, CoA, diethiothreitol, N-acetylcysteine (NAC), rat liver glutathione S-transferases, and γ-glutamyltranspeptidase were purchased from Sigma Chemical Co. (St. Louis, MO). Triethyramine, ethyl chloroformate, potassium bicarbonate, and THF (anhydrous) were purchased from Aldrich Chemical Co. (Milwaukee, WI). CA-SCoA and CA-SG derivatives were synthesized as described below. All solvents used for HPLC were of chromatography grade.

**Instrumentation and Analytical Methods.** HPLC was carried out on a Shimadzu LC-600 isocratic system coupled to a Shimadzu SPD-6AV UV-visible detector (Kyoto, Japan). All HPLC analyses were performed on a reverse-phase column (Alltech Econosil C18; 25 cm × 4.6 mm; 5 μm). Tandem liquid second-ary-ion mass spectrometry (LSIMS) was performed on a Kratos Concept IIHH four-sector tandem mass spectrometer (Kratos Analytical Instruments, Chestnut Ridge, NY) equipped with a cesium ion source and a continuous flow LSIMS probe. The solvent used contained acetonitrile (5%), thioglycerol (2%), and trifluoroacetic acid (0.1%) in water. Samples were delivered at a flow rate of 3 μl/min using a syringe pump (Applied Biosystems, Foster City, CA). CID of the 12 C component of the protonated molecules of interest was performed in the collision cell. Electrospray mass spectrometry of S-acyl-glutathione and S-acyl-NAC derivatives were performed on a Hewlett Packard 1100 LCMSD bench-top electrospray mass spectrometer (Palo Alto, CA) at a fragmentor voltage of 130 by direct infusion of the sample, dissolved in methanol/1% acetic acid (50:50), into the ion source (25 μl/min).

1H NMR spectra were recorded (in 2H6-dimethyl sulfoxide) with a General Electric QE300 spectrometer (Fairfield, CT) operating at 300 MHz. Proton chemical shifts are reported in parts per million (ppm; 8 relative to the residual dimethyl sulfoxide line.

**Synthesis of CA-SCoA.** Synthesis of CA-SCoA was performed by conventional procedures using ethyl chloroformate (Stadtman, 1957). Briefly, to CA (1.6 mmol) dissolved in anhydrous THF (25 ml) was added, at room temperature and while stirring, triethylamine (1.6 mmol) followed by ethyl chloroformate (1.6 mmol). After 30 min of continued stirring, the precipitate that formed (triethylamine hydrochloride) was removed by passing the reaction mixture through a glass funnel fitted with a glass wool plug. The filtered mixture was then added to a solution containing CoA (0.13 mmol, 100 mg) and KHCO3 (1.6 mmol) in distilled water (10 ml) and THF (15 ml). The solution was stirred continuously under nitrogen gas at room temperature for 2 h, after which the reaction was terminated by the addition of concentrated HCl (8 drops). The THF was then removed by evaporation under reduced pressure, and the remaining aqueous phase extracted with diethyl ether (4 × 50 ml). Residual diethyl ether was removed by evaporation under reduced pressure at room temperature. The solution was adjusted to pH 7.0 by the addition of NaOH (1 N). Purification of CA-SCoA was achieved by reversed-phase HPLC using isocratic elution with acetonitrile (20%) in 0.2 M ammonium acetate on a reverse-phase column (C18; 25 cm × 4.6 mm; 5 μm; 1 ml/min) and detected by UV absorbance (262 nm). CA-SCoA-containing fractions were collected and desalted by passing through a cation-exchange solid-phase extraction cartridge (J.T. Baker, Phillipsburg, NJ). Desalted solutions containing CA-SCoA were then frozen (−80°C) and lyophilized to dryness. MS/MS (CID of MH+ at m/z 964, 100%; m/z 508 [adenosine triphosphate + 2H+]), 457 ([M + H − adenosine triphosphate]+, 35%), 428 [adenosine diphosphate + 2H+], 8%, 355 ([M + H − 609]+, 6%) 169 ([p-CI-C6H4OC(CH3)3], 7%), and 136 [adenine + H]+, 25%.

**Synthesis of S-Acyl-Glutathione Thioesters.** The synthesis of glutathione thioesters was accomplished by the same method as described above for the synthesis of CA-SCoA, except that CoA was replaced by GSH (500 mg). Acyl-glutathione conjugates were prepared from clofibrate acid, gemfibrozil, carprofen, naproxen, fenoprofen, diclofenac, zomepirac, tolmetin, 4-chlorophenoxyacetic acid, 2,4-chlorophenoxyacetic acid, and 2,3-dichlorophenoxyacetic acid by this method. After the first evaporation step and subsequent...
extracts with diethyl ether, a white precipitate, the acyl-glutathione conjugate, was formed. This precipitate was washed with distilled water (4 × 50 ml) to remove remaining GSH and then by acetone (4 × 50 ml) to wash away remaining free acid and water. Finally, the precipitate was dried under a stream of nitrogen gas at room temperature (1 h). LSIMS tandem mass spectrometric and NMR analysis were performed for CA-SG: MS/MS (CID of \[\text{MH}^+\] at m/z 504, 100%); m/z 429 \([\text{M} + \text{H} + \text{Gly}]^+\), 4%, 375 \([\text{M} + \text{H} + \text{pyroglutamic acid}]^+\), 5%, 308 \([\text{GSH}]^+\), 6%, 272 \(([p\text{-Cl-C}_6\text{H}_4\text{OC(CH}_3)_2\text{COSCH}_3\text{CH}_2=\text{NH}]^+\), 7%), 219 (7%), 169 \(([p\text{-Cl-C}_6\text{H}_4\text{OC(CH}_3)_2\text{H}]^+\), 95%), 130 \(([p\text{-pyroglutamyl acid + H}]^+\), 11%), 84 \(([p\text{pyroglutamyl acid - CO}_2\text{H}]^+\), 18%), and 76 \([\text{Gly + H}]^+\), 14%). 1 H NMR (\(2 H_6\)-dimethyl sulfoxide): \(6.142 \text{ (s, 6H, -C(CH}_3)_2\text{-CH}^\text{=NH}]\), 2.28 (m, 2H, Glu-\(\beta\)-\(\beta\)), 2.5 (m, 2H, Glu-\(\gamma\)-\(\gamma\)), 2.95 (m, 2H, Cys-\(\beta\)-\(\beta\)), 3.67 (t, 2H, J = 6.4 Hz, Glu-\(\alpha\)), 3.72 (s, 2H, Gly-\(\alpha\)-\(\alpha\)), 4.46 (m, 2H, Cys-\(\gamma\)), 6.9–7.4 (m, 4H, Ar). All other synthetic S-acyl-glutathione conjugates were analyzed by reverse-phase HPLC and electrospray LC/MS, as described above (Table 1).

Synthesis of S-Acyl-NAC Thioesters. S-Acyl-NAC conjugates were obtained by reacting S-acyl-glutathione conjugates (1 mM) with NAC 20 (mM) in buffer (pH 7.4, 37°C) in a volume of 10 ml until the reaction was complete, as indicated by HPLC analysis of the reaction mixture. The incubation then was acidified to pH 3 by the addition of 1 mM HCl, followed by extraction of the S-acyl-NAC conjugate with ethyl acetate (1 × 10 ml). The ethyl acetate layer was then dried (anhydrous MgSO\(_4\)) and evaporated to afford product. S-Acyl-NAC derivatives then were analyzed by ESIMS, as described above for CA-SG (Table 1).

Incubation Conditions. Incubations of CA-SG with GSH were described as in similar experiments with 1-O-CAG (Shore et al., 1995), briefly detailed as follows: in general, incubations (a 0.5-ml total volume) included CA-SCoA (1 mM), GSH (5 mM), and EDTA (5 mM) in 0.05 M potassium phosphate buffer (pH 7.5) and were performed at 37°C in screw-capped glass vials in a shaking incubator. Aliquots (5 \(\mu\)l) of the incubation mixture were taken at the indicated time points and added to the incubation buffer (250 \(\mu\)l). An aliquot (100 \(\mu\)l) of this solution then was injected immediately onto the HPLC for the analysis of reaction products. HPLC analysis was performed on a reverse-phase column (see above) with a mobile phase of 50% methanol in 0.05 M potassium phosphate in distilled water (pH 4.2). CA, CA-SCoA, and CA-SG were detected by UV absorbance at 226 nm. Quantitative measurements were made using a standard curve generated from absolute peak areas.

pH-Dependent Nonenzymatic Hydrolysis of CA-SCoA. The nonenzymatic hydrolysis of CA-SCoA (1 mM) buffer at 37°C was characterized in incubations, as described above but at varying pH (2, 3, 4, 6, 7, 8, 9, 10, 10.5, 11, 11.5, 12, and 12.5). Indicated pH values were attained by adjusting the pH of 0.05 M potassium phosphate solution with HCl (1 M) or NaOH (1 M). Incubations were analyzed after 1 and 24 h for the loss of CA-SCoA and the increase in formation of CA by HPLC methods, as described above. Further experiments were performed to determine the time-dependent hydrolysis of CA-SCoA at pH 7.5 and 37°C, with analysis of CA-SCoA remaining after 1, 2, 3, 4, and 5 days of incubation.

Enzymatic Catalysis by GSTs. GST catalysis of the transacetylation reaction of CA-SCoA with GSH was evaluated by incubating CA-SCoA with GSH (as above) in the presence of rat liver GSTs (1–4 mg/ml) and analyzing the rate of CA-SG formation over time by HPLC methods, as outlined above.

Enzymatic Cleavage of CA-SG. Solutions containing CA-SG, formed in vitro by the reaction of CA-SCoA with GSH, were incubated in the presence or absence of \(\gamma\)-glutamyltranspeptidase (0.1 unit) in incubation buffer (as above) at 37°C for 1 h. One unit of the transpeptidase will liberate 1 mmol of \(p\)-nitroaniline from \(L\)-\(\gamma\)-glutamyl\(p\)-nitroaniline per minute at pH 8.5 and 25°C. Aliquots of the incubation were analyzed by HPLC (as above) for the loss of CA-SG and respective formation of clofibrin-cysteinylglycine. Confirmation of the formation of clofibrin-cysteinylglycine was obtained by tandem mass spectrometric analysis, as described above.

Reaction of CA-SCoA with DTT. Incubation of dithiothreitol (DTT; 5 mM) with CA-SCoA (1 mM) and subsequent analysis of the reaction products by HPLC were performed at 37°C using methods similar to those described above for the reaction of CA-SCoA with GSH. HPLC-purified products of the reaction were characterized by LSIMS tandem mass spectrometry, as described above.

Reaction of S-Acyl-Glutathione Conjugates with NAC. S-Acyl-glutathione conjugates (0.1 mM) were incubated with NAC (1 mM) in buffer (0.05 M KHCO\(_3\), pH 7.5) at room temperature and aliquots (20 \(\mu\)l) of the incubation mixture were analyzed directly by HPLC (as above for CA-SG) for the formation of S-acyl-NAC products. Quantitative measurements were made using a standard curve generated from absolute peak areas of the respective synthetic S-acyl-NAC derivatives.

### Table 1

<table>
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<th>S-Acyl-Glutathione Derivatives</th>
<th>MH^+</th>
<th>[MH^+−75]^+</th>
<th>[MH^+−129]^+</th>
<th>[MH^+−232]^+</th>
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<td>488</td>
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<td>331</td>
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<td>391</td>
<td>288</td>
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<tr>
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<td>457</td>
<td>403</td>
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<td>381</td>
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N.D. not detected.
Results

HPLC Analysis of CA-SCoA, CA, and CA-SG. The analysis of products formed during incubations of CA-SCoA with buffer in the absence or presence of GSH was performed by reverse-phase isocratic HPLC (Fig. 2), which allowed for the separation of CA-SCoA (4.5 min), CA (5.0 min), and CA-SG (5.5 min).

Chemical Stability of CA-SCoA. Analysis of the chemical stability of CA-SCoA toward hydrolysis of the thioester bond in buffer at 37°C and pH 7.5 showed that the acyl-CoA derivative is chemically stable under physiological conditions with a long apparent half-life of ~21 days (data not shown). Studies of the stability of CA-SCoA in buffer performed at 37°C and varying pH (Fig. 3) demonstrated that the thioester is very stable in acidic and basic aqueous solutions from pH 2 to pH 10. Under these pH conditions and after a 1-day incubation, the incubation mixtures showed minimal loss of CA-SCoA or appearance of CA. Although in solutions having pH values greater than 10, CA-SCoA exhibits hydrolysis (Fig. 3). Approximately 50% liberation of free CA occurs after 1 h at pH 11.5 and after 24 h at pH 10.5. CA-SCoA was found to undergo complete hydrolysis to CA after a 1-h incubation at pH 12.5 and 37°C.

Reaction of CA-SCoA with GSH. After 24 h of incubation of CA-SCoA (1 mM) with GSH (5 mM) in pH 7.5 buffer at 37°C, a substance was formed that coeluted with the synthetic CA-SG standard (Fig. 4). Similar incubations in the absence of GSH showed no material eluting at the HPLC retention time of CA-SG. CA-SG purified by HPLC from the incubation was identified on the basis of its LSIMS tandem mass spectrum (Baillie and Davis, 1993), which was identical to that of the corresponding synthetic standard (Fig. 5). CID of the parent MH⁺ ion (m/z 504) of both the in vitro-purified product and the standard CA-SG showed product ions at m/z 429 ([MH⁺-Gly⁺⁺], m/z 375 ([MH⁺-pyroglutamic acid⁺⁺]), m/z 357 ([375-H₂O⁺⁺], m/z 308 ([GSH⁺⁺]), m/z 272 ([p-Cl-C₆H₄OC(CH₃)₂COSCH₂CH=NH₂⁺⁺]), m/z 169 ([p-Cl-C₆H₄OC(CH₃)₂⁺⁺]), m/z 130 ([pyroglutamic acid + H⁺]), m/z 84 ([pyroglutamic acid-CO₂H⁺⁺]), and m/z 76 ([Gly + H⁺]). Further evidence on the structure of CA-SG formed in vitro came from studies with γ-glutamyltranspeptidase. When γ-glutamyltranspeptidase was incubated with CA-SG formed in vitro and products of the reaction were analyzed by reverse-phase HPLC, a new product was formed that eluted after CA-SG, with a retention time of 9.5 min (data not shown). Analysis of the purified reaction product by LSIMS tandem mass spectrometry gave a product ion spectrum (Fig. 6) consistent with clofibaryl-5-acyl-cysteinylglycine (CA-SCG). CID of the parent MH⁺ ion (m/z 375) of the HPLC-purified CA-SCG showed ions at m/z 357 ([MH-H₂O⁺⁺], m/z 331 ([MH⁺-CO₂H⁺⁺]), m/z 300 ([MH⁺-Gly⁺⁺]), m/z 272 ([p-Cl-C₆H₄OC(CH₃)₂COSCH₂CH=NH₂⁺⁺]), m/z 169 ([p-Cl-C₆H₄OC(CH₃)₂⁺⁺]), and m/z 76 ([Gly + H⁺]), which are consistent with its structure. The tandem mass spectra of the CA-SG formed in vitro and of the γ-glutamyltranspeptidase degradation product provide strong evidence that the site of acylation is the nucleophilic cysteinyl thiol of GSH in the transacylation reaction and not the free amine of the glutamic acid moiety.

Time-Dependent Reaction of CA-SCoA with GSH. The reaction of CA-SCoA with GSH in vitro (pH 7.5, 37°C) resulted in a time-dependent linear formation of CA-SG during the first 4 h of incubation (~50 µM/h); after 21 h, 0.7 mM CA-SG was formed (Fig. 7).
Incubation Studies in the Presence of GST. To evaluate the ability of GSTs to catalyze the transacylation of GSH by CA-SCoA in vitro, incubations of CA-SCoA with GSH were performed both in the absence and presence of rat liver GSTs, and the products were analyzed by HPLC after 0, 1, 2, and 3 h of incubation. The rate of transacylation of GSH by CA-SCoA increased ~3-fold in the presence of 4.0 mg/ml GST and was GST concentration-dependent (Fig. 8). Functional enzyme was necessary for catalysis because denaturation of GST by heating completely abolished any enzyme-catalyzed increase in transacylation of GSH by CA-SCoA (data not shown).

Reaction of S-Acyl-Glutathione Derivatives with NAC. A range of S-acyl-glutathione derivatives were synthesized and used in in vitro studies to compare their abilities to transacylate the nucleophilic thiol of NAC. ESI/MS analysis of the synthetic S-acyl-glutathione conjugates gave spectra that were consistent with their structures (Table 1; Fig. 9). ESI/MS was performed on the S-acyl-glutathione thioester derivatives in the positive-ion mode. Each acyl-glutathione derivative afforded intense MH⁺ ions. Fragmentation of the parent MH⁺ ions gave a similar pattern of fragment ions for each analyte. Three major ions exist in the spectra of the thioester glutathione derivatives. The m/z values of the ions corresponding to [MH⁺-232]⁺, [MH⁺-129]⁺, and [MH⁺-75]⁺ are of particular interest because they reflect the mass of the acyl group. Incubation of S-acyl-glutathione derivatives with NAC led to the formation of the respective S-acyl-NAC conjugates at a rate that was dependent on the substitution at the ω-carbon of the acyl-linkage. The rank order of reactivity of the S-acyl-glutathione derivatives with NAC was phenoxyacetyl- > ary lacetyl- > 2-phenylpropionyl- = α,α-dimethyl-phenoxyacetyl- > α,α-dimethyl-substituted S-acyl-glutathione. Clofibryl-S-acyl-glutathione was shown to react with NAC to form clofibryl-S-acyl-NAC in a time-dependent and linear fashion. The identity of the formed CA-SNAC was confirmed by ESI/MS (Fig. 10). Reaction of the remaining S-acyl-glutathione derivatives with NAC gave S-acyl-NAC conjugates in which the structures were also confirmed by ESI/MS (Table 1).

Reaction of CA-SCoA with DTT. Further experiments were performed on the reaction of CA-SCoA with DTT, a reducing agent with nucleophilic thiol groups. Incubation of DTT (5 mM) with CA-SCoA (1 mM) led to the time-dependent transacylation of DTT as detected by HPLC to give clofibryl-S-acyl-DTT (Fig. 11). Characterization
of the HPLC-purified product clofibryl-S-acyl-DTT by LSIMS tandem mass spectrometry gave a mass spectrum consistent with its structure (Fig. 12). CID of the parent MH$^+$ (m/z 351) showed ions at m/z 333 ([MH$^+$-H$_2$O]$^+$), m/z 315 ([MH$^+$-2H$_2$O]$^+$), m/z 243 ([p-Cl-C$_6$H$_4$OC(CH$_3$)$_2$-CO-S-CH$_2$]$^+$), m/z 197 ([MH$^+$-DTT]$^+$), m/z 169 ([p-Cl-C$_6$H$_4$OC(CH$_3$)$_2$]$^+$), and m/z 127 ([p-Cl-C$_6$H$_4$O]$^+$).

### Discussion

Chemically reactive intermediates formed during the metabolism of acidic drugs are believed to mediate their toxic side effects (Boelsterli et al., 1995). These compounds include many agents used clinically, such as carboxylic acid-containing nonsteroidal anti-inflammatory drugs and hypolipidemic drugs (Faed, 1984). The mechanisms responsible for the initiation of toxic side effects, which includes severe and sometimes fatal immune reactions, remain poorly understood. Increasing evidence suggests that carboxylic acid-containing drugs are metabolized to reactive metabolites that react with proteins irreversibly (Spahn-Langguth and Benet, 1992). Two routes of metabolism for carboxylic acid-containing drugs are acyl glucuronidation and acyl-CoA formation, both of which may lead to the generation of reactive metabolites capable of binding irreversibly to protein (Fig. 1). Clofibric acid is metabolized by both acyl glucuronidation (Emudianughe et al., 1983) and acyl-CoA formation (Lygre et al., 1986) and covalently binds to proteins in exposed individuals (Sallustio et al., 1991). Previous studies have shown that 1-O-CAG may react with the nucleophilic cysteinyl thiol of GSH in a transacylation-type fashion to...
form CA-SG thioester (Shore et al., 1995). The transacylation reaction, which was conducted in buffer at 37°C and pH 7.5 in the presence of 1 mM 1-O-CAG and 5 mM GSH, resulted in the formation of CA-SG (1.3 μM/h) as a minor product. The major reaction occurring in that study (Shore et al., 1995) was intramolecular acyl migration of 1-O-CAG (50% remaining at 3 h), forming isomers that were determined to be unreactive with GSH. The competing intramolecular acyl migration reaction may be functioning to divert the unstable 1-acyl glucuronide from acylation of nucleophiles in vivo.

In the present study, experiments were conducted to evaluate the chemical reactivity of CA-SCoA with GSH to determine the acylating properties of this thioester derivative. Initial experiments evaluating the hydrolysis of CA-SCoA in buffer at pH 7.5 and 37°C showed that the thioester was slowly hydrolyzed (~2% hydrolysis/day). This result demonstrates that CA-SCoA is relatively stable in buffer under physiological conditions compared with the degradation reactions that occur for 1-O-CAG under identical conditions (Bailey and Dickinson, 1996). During incubations performed with GSH (5 mM) and CA-SCoA (1 mM) (Fig. 4), as in previous studies on the reaction of 1-O-CAG with GSH (Shore et al., 1995), we found that CA-SCoA reacts, in a transacylation-type fashion, with the nucleophilic cysteinyl thiol of GSH to form CA-SG (50 μM/h) (Fig. 7). The rate at which CA-SG is formed here is approximately 40-fold greater than was determined in experiments with 1-O-CAG, presumably due to the competing intramolecular acyl migration reactions of the 1-O-acyl glucuronide, resulting in the formation of stable conjugates. Tandem mass spectrometric analysis of the CA-SG formed here confirmed the structure of CA-SG (Fig. 5) in that the product ions formed were consistent for the glutathione conjugate (Baillie and Davis, 1993), having a thioester linkage with CA. As in studies on the reactivity of 1-O-CAG with GSH, our analysis of the products formed on reaction of CA-SCoA with GSH showed no evidence for the formation of amide conjugates from acylation of the glutamyl-amine of GSH. Further evidence came from degradation studies with γ-glutamyltranspeptidase that resulted in the formation of a glutathione conjugate not containing glutamic acid. Characterization of the identity of the CA-S-acetyl-cysteinylglycine conjugate was determined by tandem mass spectrometric analysis (Fig. 6). The result showing that CA-SG is a substrate for γ-glutamyltranspeptidase was not surprising since the corresponding mercapturic acid conjugate has been isolated from the urine of CA-dosed patients (Stogniew and Fenselau, 1982). γ-Glutamyltranspeptidase-mediated degradation of S-acyl-glutathione derivatives was previously demonstrated by Tate (1975). Products of this degradation reaction undergo rearrangement to form drug-N-acyl-cysteinylglycine amides. Because of the steric hindrance of the α-methyl groups on CA, this rearrangement may not be occurring in incubations with γ-glutamyltranspeptidase. Our MS/MS spectra do not differentiate between the isobaric isoforms clofibryl-S-acyl- and clofibryl-N-acyl-cysteinylglycine (Fig. 6).

Because it has been shown that 1-O-CAG is a substrate for GST (Shore et al., 1995), we investigated the possibility that CA-SCoA could also be a substrate for the enzyme. When CA-SCoA was incubated with rat liver GST, a 3-fold increase in the rate of formation of the glutathione conjugate was found (Fig. 8). To our knowledge, this is the first data showing that an acyl-CoA derivative is a substrate for GST. The importance of GST in catalyzing the conjugation of GSH to reactive xenobiotic-S-acyl-CoA derivatives is not yet known, but it may provide a detoxification mechanism similar to that proposed for amino acid conjugation reactions that occur for a number of xenobiotic acyl-CoA conjugates (Caldwell, 1984).

Structure activity relationship studies have shown that the degree of substitution at the α-carbon of carboxylic acid drugs can be used to predict the extent to which their respective 1-acyl glucuronides undergo hydrolysis, acyl migration, and covalent binding to protein in vitro and in vivo (Benet et al., 1993). We propose that the same structure activity relationship would exist for the ability of S-acyl-CoA metabolites to acylate protein. Experiments were performed in which a range of S-acyl-glutathione derivatives were studied, instead of the respective acyl-S-CoA thioesters. We assumed that the reactivity of the S-acyl-glutathione derivative with nucleophiles in vitro would be essentially the same as that of the respective S-acyl-CoA thioesters. Incubation of S-acyl-glutathione derivatives with NAC resulted in the formation of the respective S-acyl-NAC conjugates at a rate that was dependent on the substitution at the α-carbon of the acyl-linkage (Fig. 9). The rank order of reactivity of the S-acyl-glutathione derivatives with NAC was phenoxacyetyl- > arylocetyl- > 2-phenylpropionyl- = α,α-dimethyl-phenoxacyetyl- > α,α-dimethyl-substituted S-acyl-glutathione, which is directly analogous to relative degradation rates known for acyl glucuronides. The presence of an oxygen atom at the β-position, rather than a methylene group as found in the phenoxacetic acids and clofibric acid, results in more reactive acylating species, which may be due to the inductive effects of the oxygen atom. These data show that S-acyl-glutathione thioesters are reactive in transacylation-type reactions with thiols and, therefore, may also contribute to the overall covalent binding to thiols in vivo. Subsequent degradation of S-acyl-glutathione derivatives to the corresponding mercapturic acid conjugates in vivo would result in the formation of S-acyl-NAC thioesters that are presumably reactive acylating species as well and could covalently bind to tissue nucleophiles. Intramolecular rearrangement of the γ-glutamyltranspeptidase S-acyl-cysteinylglycine intermediate (Tate, 1975), forming an unreactive amide, may provide a mechanism for detoxification reactive S-acyl-glutathione products.

In experiments performed to assess the influence of acyl-CoA formation on the extent of acylation of protein in vitro, the acidic drug of interest is incubated in the presence of CoA, ATP, Mg2+, and the nucleophilic reducing agent DTT. In previously reported studies with diclofenac (Hargus et al., 1994), the importance of acyl-CoA formation has been overlooked because in such studies the covalent binding of the acidic drug to protein was not detected. We propose that the presence of millimolar amounts of DTT, although often necessary for the formation of acyl-CoA thioesters, would compete with protein nucleophiles for covalent binding. In the present studies, DTT (5 mM) was shown to react with CA-SCoA (1 mM) almost quantitatively in
less than a 4-h incubation at pH 7.5 and 37°C to form CA-acyl-DTT (Fig. 11). The rate of this reaction was much faster than the rate of a reaction in similar incubations with GSH (Fig. 7), indicating that DTT is a superior nucleophile in transacylation reactions with acyl-CoA derivatives. The product CA-acyl-DTT, which contains adjacent hydroxyl groups (Fig. 12), may undergo an intramolecular acyl migration, similar to that seen for acyl glucurononides, to more stable oxygen ester products. Although this migration reaction was not investigated in the present article, it would provide an explanation for the increase in reaction rates versus experiments with GSH by making the reaction less reversible through the formation of stable oxygen esters. However, tandem mass spectrometric analysis of the acylated DTT product gave a mass spectrum consistent with the product ion at -acyl-DTT (Fig. 12), as indicated by the presence of the S-acyl-DTT product ion, which gave a mass spectrum consistent with the product ion of the reaction less reversible through the formation of stable oxygen esters. We propose that CA-SCoA can transacylate DTT, and we further suggest that CA-SCoA is more reactive than its respective acyl glucuronide in transacylation-type reactions, in competition with and reduced the level of nafenopin-protein adduct formation.

In conclusion, results from these in vitro studies indicate that acyl-CoA thiester derivatives of acidic drugs are reactive acylating species that, in addition to acyl glucurononides, may be responsible for protein acylation in vivo. Furthermore, studies with clotibior-β-acyl-CoA thiester have shown that clotibior-β-acyl-CoA thiester is more reactive than its respective acyl glucurononide in transacylation-type reactions with glutathione. Depending on the amounts formed and the sites of formation, the contribution of these two metabolites in the covalent binding of drug to proteins would vary. Ongoing studies in our laboratory are designed to characterize the chemical reactivity of a number of xenobiotic β-acyl-CoA derivatives, compare this chemical reactivity with the corresponding acyl glucurononides, elucidate the mechanisms by which protein acylation may occur for carboxylic acid-containing drugs through the CoA thiester metabolite, and evaluate the immunotoxic potential of β-acyl-CoA thiester derivatives.

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