Biochemical Pharmacology, Vol. 42, No. 7, pp. 1421–1425, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

IN VIVO COVALENT BINDING OF CLOFIBRIC ACID TO HUMAN PLASMA PROTEINS AND RAT LIVER PROTEINS

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(*Received* 11 *March* 1991; *accepted* 30 *May* 1991)

Abstract—Recent studies have shown that acyl-glucuronide conjugates are chemically reactive electrophilic metabolites that can undergo transacylation reactions resulting in intra-molecular rearrangement, hydrolysis and covalent binding of aglycone to albumin both *in vitro* and *in vivo*. The hypolipidaemic agent clofibrate is eliminated almost entirely as clofibric acid glucuronide in humans and rats. The formation of clofibric acid–protein adducts was investigated in 14 patients receiving 0.5–2.0 g/day of clofibrate for hypercholesterolaemia, and in liver homogenates from 20 rats administered 280 mg/kg/day of clofibric acid for up to 21 days. Total clofibric acid concentrations in the patients ranged from 0 to 114 mg/L. Covalently bound clofibric acid–protein adducts were detected in all patients, even in one subject in whom there was no measurable plasma clofibric acid. Concentrations ranged from 2.2 to 53.4 ng/mg protein and, in eight patients receiving 1.0 g/day of clofibrate, were correlated ($P < 0.05$) with renal function as assessed by creatinine clearance. Clofibric acid–protein adducts were also present in rat liver homogenates, and increased with increasing duration of treatment ($P < 0.0001$), from a mean (SE) of 10.1 (0.7) to 32.3 (1.6) ng/mg protein. The covalent binding of drugs to tissue macromolecules has traditionally been associated with toxicity. Further research is required to elucidate the role of acyl-glucuronide conjugates in the formation of drug–protein adducts and their biological consequences.

Clofibrate, the ethyl ester of *p*-chlorophenoxyisobutyric acid, is used clinically in the treatment of hyperlipoproteinemias. *In vivo*, clofibrate is rapidly and completely hydrolysed to clofibric acid (*p*-chlorophenoxyisobutyric acid), the pharmacologically active form, and no unchanged clofibrate is detectable in blood or urine [1]. It has been suggested that the clinical use of clofibrate is associated with an increased incidence of liver, gastro-intestinal and kidney disease [2]. In rats, clofibric acid produces alterations in hepatic function including peroxisomal proliferation [3]. In both man [4] and rats [4, 5] clofibric acid is eliminated almost entirely (>90%) as clofibric acid glucuronide. Recent studies have shown that the glucuronide conjugates of carboxylic acids are chemically reactive metabolites, due to the presence of the unsaturated carbonyl group which is susceptible to nucleophilic substitution [6]. However, the role of these reactive acyl-glucuronide conjugates in general, and of clofibric acid glucuronide, in clinical toxicity is as yet uknown.

The simplest example of nucleophilic substitution is hydrolysis by the nucleophilic hydroxyl ion, thus regenerating the parent carboxylic acid. Hydrolysis has been demonstrated both *in vivo* and *in vitro* for a number of acyl-glucuronide conjugates [6], including those of clofibric acid [7]. Consequently acyl-glucuronide formation is a readily reversible process with several important pharmacokinetic and toxicological consequences [8]. Nucleophilic substitution can also occur with the hydroxyl groups on the glucuronic acid moiety of a conjugate, resulting in intramolecular rearrangement of the 1-*O*-acyl-glucuronide, so that the aglycone becomes attached at the C-2, -3 or -4 positions of the glucuronic acid molecule. Such rearrangement has been demonstrated for many acyl-glucuronides [6] including clofibric acid glucuronide [9]. In addition, acyl-glucuronides are also susceptible to nucleophilic substitution by sulfhydryl compounds [10]. van Breemen and Fenselau [11] first demonstrated that nucleophilic substitution of acyl-glucuronide conjugates could occur via sulfhydryl groups in proteins, such as albumin, resulting in the covalent binding of the aglycone molecule to the protein. Since then the *in vivo* acylation of human plasma proteins has been documented for zomepirac [12], tolmetin [13], carprofen [14], diflunisal and probenecid [15], and it has been suggested that the presence of these circulating xenobiotic protein adducts may be associated with the high incidence of anaphylactic reactions associated with the clinical use of many non-steroidal anti-inflammatory drugs [12–14].

A previous study has reported that clofibric acid glucuronide concentrations are undetectable in the plasma of human volunteers administered clofibrate [16]. However, clofibric acid glucuronide has been shown to mediate the formation of covalently bound clofibric acid–albumin adducts *in vitro* [11]. In this study we investigated the *in vivo* formation of covalently bound clofibric acid–plasma protein adducts in man. An additional study was also carried out in rats to examine the formation of covalently bound clofibric acid–protein adducts in the liver, which is the primary site of clofibric acid glucuronide

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synthesis and would therefore be exposed to large quantities of glucuronide conjugate.

MATERIALS AND METHODS

Clofibric acid was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and the glucuronide conjugate biosynthesized as described previously by Veenendaal and Meffin [17]. All other chemicals used were of the highest analytical grade.

Plasma samples were obtained from 14 patients (4 male, 10 female), ranging in age from 35 to 73 years, who required monitoring of plasma clofibric acid concentrations. All were receiving between 0.5 and 2.0 g/day p.o. of clofibrate (Atromid, ICI Australia) for hypercholesterolaemia, and had been on a constant dose for at least 1 month. A single trough blood sample was taken from each patient at approximately 12 or 24 hr post dose, and the plasma was stored at −20° until analysed.

Twenty adult male Hooded Wistar rats (250–300 g) were administered 280 mg/kg/day p.o. of clofibric acid in methylethyl cellulose vehicle and groups of five animals were killed following 1, 2, 10 and 21 days of dosing. A further eight rats, used as controls, were administered the methylethyl cellulose vehicle only (5% w/v) and two animals were killed, in parallel with the treated animals, at 1, 2, 10 and 21 days of dosing. The livers were immediately removed, perfused, homogenized with ice-cold buffer (10 mM phosphate containing 1.15% KCl, pH 7.4) and centrifuged at 700 *g* for 10 min. The supernatant was assayed for protein [18].

The formation of clofibric acid–protein adducts was measured using the method of Smith *et al.* [12]. Briefly, to 1 mL of plasma or liver homogenate were added 1 mL of ice-cold methanol, 2 mL of acetonitrile and 10 μL of *ortho*-phosphoric acid, with thorough mixing following each addition. The sample was then centrifuged to pellet the precipitate and the supernatant was discarded. The protein pellet was washed nine times with a 6 mL mixture of methanol/diethyl ether (3:1) to remove either non-covalently bound clofibric acid or metabolites. Finally the pellet was dissolved in 1 N KOH and heated at 80° for 3 hr to liberate covalently bound clofibric acid. Samples were then acidified by adding 1 mL distilled water and 90 μL *ortho*-phosphoric acid, after which 75 μL of internal standard (probenecid, 6 mg/L) and 5 mL of dichloromethane were added. Together with a series of calibration standards (0.1–2.0 μg), the samples were mixed for 10 min, centrifuged, and the organic layer was collected and evaporated to dryness at 40° under nitrogen. The amount of clofibric acid–protein adduct present was expressed as nanograms of clofibric acid released following hydrolysis of the protein pellet (i.e. ng/mg protein).

Liberated clofibric acid from covalently bound adducts, and non-covalently bound clofibric acid in human plasma or rat liver homogenates was measured using the HPLC method of Veenendaal and Meffin [17]. A series of blank plasma or blank homogenate samples to which clofibric acid (100 or 1000 mg/L) or clofibric acid glucuronide (100 or 1000 mg/L) were added just prior to protein precipitation were also included in each analysis to ensure that there was no interference from residual drug or metabolite not removed by the methanol/diethyl ether washes.

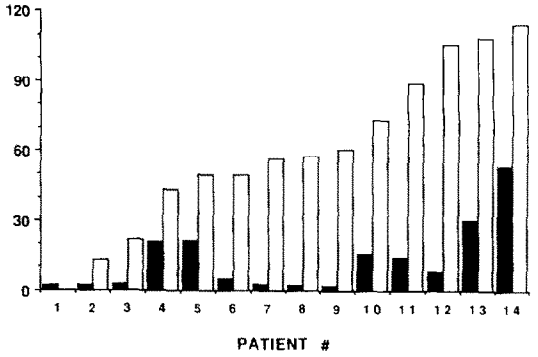

Fig. 1. Covalently bound clofibric acid–protein adduct (■ ng/mg protein), and non-covalently bound clofibric acid (□ mg/L) present in the plasma of 14 patients. The daily dose of clofibrate and approximate sampling times (relative to dose) for each patient are as follows: patients 4, 13, 14—2.0 g/day at 12 hr; patients 3, 5, 10, 11, 12—1.0 g/day at 12 hr; patients 1, 2, 6—1.0 g/day at 24 hr; patient 7—0.75 g/day at 12 hr; patients 8, 9—0.5 g/day at 12 hr.

Plasma creatinine concentrations for the human volunteers were measured using a standard rate reaction Jaffe method by the Department of Clinical Chemistry at The Queen Elizabeth Hospital. Creatinine clearance was calculated from plasma creatinine concentrations according to the method of Cockcroft and Gault [19], taking into account patients' sex, age and weight.

Linear regression analysis was carried out comparing creatinine clearance with plasma protein adduct formation in eight patients who were all receiving 1.0 g/day of clofibrate, and also to confirm the effect of age on creatinine clearance in all 14 patients. One-way analysis of variance was used to compare the duration of clofibric acid dosing with the amount of clofibric acid or protein adduct formation in rat liver.

RESULTS

Total plasma clofibric acid concentrations in the human patients ranged from 0 to 114 mg/L, and covalently bound clofibric acid–protein adducts were detected in the plasma of all patients, including one subject in whom there was no measurable plasma clofibric acid (Fig. 1). The concentrations of clofibric acid–protein adducts ranged from 2.2 to 53.4 ng/mg protein. Creatinine clearances varied from 48 to 138 mL/min and were inversely correlated with patient ages (creatinine clearance = 199.4 − 1.99 × age; $r^2 = 0.83$, $P < 0.001$). There was also a significant correlation ($P < 0.05$) between creatinine clearance and the formation of clofibric acid–protein adducts in patients receiving 1.0 g/day of clofibrate (Fig. 2).

Clofibric acid–protein adducts were also present in rat liver homogenates. Adduct concentrations increased with increasing duration of treatment

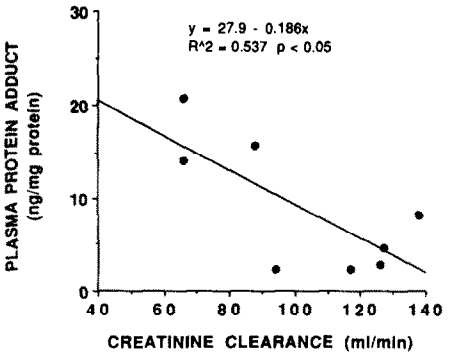

Fig. 2. Linear regression analysis of creatinine clearance vs covalently bound clofibric acid–plasma protein adduct present in eight patients receiving 1.0 g/day of clofibrate.

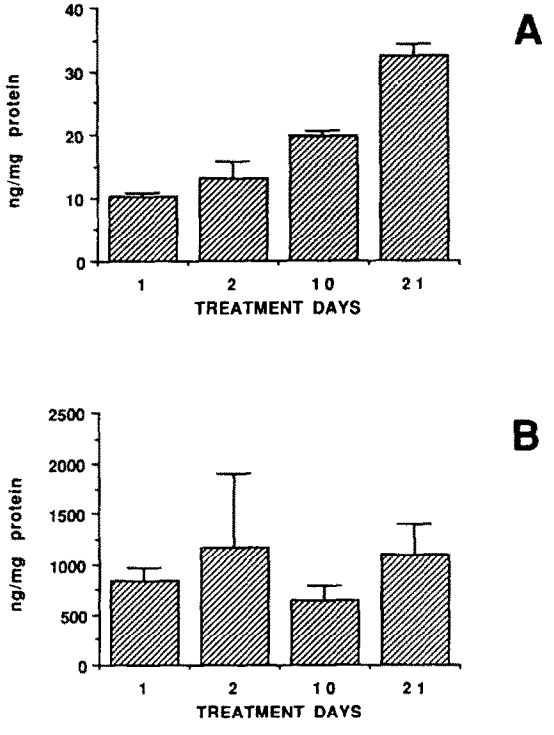

Fig. 3. Mean (± SE) liver homogenate contents of (A) covalently bound clofibric acid–protein adduct and (B) non-covalently bound clofibric acid (representing parent drug plus metabolites), in rats receiving oral clofibric acid for up to 21 days.

($P < 0.0001$), and ranged from a mean (SE) of 10.1 (0.7) to 32.3 (1.6) ng/mg protein on days 1 and 21 of treatment, respectively (Fig. 3A). Rat liver homogenates contained between 126 and 2220 ng/mg protein of non-covalently bound clofibric acid equivalents, representing both unchanged clofibric acid plus metabolites (acyl-glucuronide) as measured following mild alkaline hydrolysis. There was no statistically significant difference ($P = 0.681$) in the amount of non-covalently bound clofibric acid material between the four treatment groups (Fig. 3B).

In both blank plasma and blank liver homogenate samples there was no analytical interference from clofibric acid or clofibric acid glucuronide added at the time of protein precipitation, indicating that all unbound clofibric acid and metabolites were removed by the methanol/diethyl ether washes.

DISCUSSION

This study has demonstrated the *in vivo* covalent binding of a hypolipidaemic agent, clofibric acid, to human plasma proteins. The direct involvement of clofibric acid glucuronide in the formation of covalently bound clofibric acid–albumin adducts has been demonstrated *in vitro* by van Breemen and Fenselau [11]. The electrophilicity of clofibric acid glucuronide has also been confirmed by its ability to react with a standard chemical nucleophile such as 4-(*p*-nitrobenzyl)pyridine, clofibric acid glucuronide being the most reactive of several acyl-glucuronides tested [20, 21]. It is therefore likely that the *in vivo* covalent binding to human plasma proteins, demonstrated in this study, is mediated by the chemically reactive clofibric acid glucuronide, and that the process involved in the *in vivo* formation of clofibric acid–plasma protein adducts occurs even with clofibric acid glucuronide concentrations two orders of magnitude less than circulating parent clofibric acid concentrations (i.e. < 1 mg/L, Veenendaal *et al.* [16]). If the amount of clofibric acid covalently bound to plasma proteins is expressed as milligrams per litre of plasma, then the amount present in plasma is up to 5.0% of non-covalently bound clofibric acid concentrations, and may be as much as 500% of plasma clofibric acid glucuronide concentrations. It would seem therefore that renal excretion [4, 7], hydrolysis [7] and covalent binding to plasma proteins all play an important role in the clearance of clofibric acid glucuronide from plasma. In one patient no plasma clofibric acid was detectable 24 hr after the last reported dose of clofibrate, probably reflecting a compliance problem (Fig. 1). However, despite undetectable plasma clofibric acid concentrations, the patient still had measurable quantities (2.24 ng/mg protein) of clofibric acid–plasma protein adduct (Fig. 1), indicating that the half-life of the adduct is much greater than that of clofibric acid, an observation consistent with the previously reported long half-lives (10–15 days) for the plasma protein adducts of diflunisal and probenecid [15].

The data show a large degree of inter-individual variability in plasma concentrations of clofibric acid and of covalently bound plasma protein adducts. The variability in plasma clofibric acid concentrations was mostly due to the range of doses the patients were receiving, and differences in sampling times. However, given the potentially very long half-life of clofibric acid–plasma protein adducts, even a 12 hr difference in sampling should not have significantly affected the measured concentration of adduct. Therefore, inter-patient variability in adduct concentrations was most likely due to differences in dosage and, as discussed below, differences in renal function.

Clofibric acid is eliminated almost entirely by

metabolism to clofibric acid glucuronide which in turn is eliminated by the kidneys [4]. The plasma clearance of clofibric acid is reduced in patients with impaired renal function [22] consistent with the previously described acyl-glucuronide futile cycle [7, 8] which predicts that as renal clearance of acyl-glucuronide diminishes more of the conjugate becomes available for elimination via electrophilic reactions such as hydrolysis, to form the parent acid, or covalent binding to plasma proteins. As expected, renal function, measured by creatinine clearance, diminished with patient's age. In the eight patients receiving 1.0 g/day of clofibrate there was a significant correlation (Fig. 2) between creatinine clearance and adduct formation, as predicted by the acyl-glucuronide futile cycle, further supporting the role of clofibric acid glucuronide in the formation of plasma protein adducts.

It has been suggested that the *in vivo* formation of covalently bound plasma protein adducts is catalysed by albumin [23, 24], and depends on an initial reversible interaction between the acyl-glucuronide conjugate and albumin [23, 24]. At present, the binding site at which this interaction occurs is still unclear [23–25], however, there is evidence that at least the benzodiazepine binding site in albumin may be involved [23–25]. The amount of covalently bound plasma protein adducts formed by acyl-glucuronide conjugates should therefore depend on (i) the affinity of the conjugate for the appropriate binding site(s) on albumin, (ii) the amount of conjugate present in plasma and (iii) the chemical reactivity of the conjugate, all of which would differ between individual conjugates. The results of this study and of previous *in vitro* work suggest that clofibric acid glucuronide is one of the more reactive acyl-glucuronide conjugates tested so far [7, 8, 20, 21], and that binding to albumin occurs at a high affinity site [11]. Fatty acids are also known to bind reversibly to albumin and thus may alter the formation of covalently bound adducts by acyl-glucuronides [23, 24]. Therefore, it is possible that other factors such as raised circulating free fatty acid concentrations, as may occur in patients with lipid disorders, could also contribute to variability in the formation of covalently bound albumin adducts.

This study has also demonstrated the *in vivo* covalent binding of clofibric acid to rat liver proteins. Liver protein adduct formation continued to rise throughout the 21 days of treatment in contrast with the liver contents of non-covalently bound clofibric acid material, which remained unchanged over the treatment period, consistent with a much longer half-life of the protein adducts in comparison to those of clofibric acid or its glucuronide conjugate. Given the chemical reactivity of clofibric acid glucuronide and its potential for specific protein interactions in the liver (e.g. biliary transport [1, 5]), it is possible for the observed covalent binding to be mediated by clofibric acid glucuronide. However, in the liver, clofibric acid is also metabolized to clofibroyl-CoA [26], another highly reactive metabolic intermediate. Hertz and Bar-Tana [27] reported that nafenopin and benzafibrate, which are structurally related to clofibrate, acylated membrane and cytosolic liver proteins in cultured rat hepatocytes. The authors proposed that the acylation of rat hepatocyte proteins by nafenopin and benzafibrate was analogous to the covalent acylation of proteins by long-chain fatty acids and thus required prior thioesterification to the respective acyl-CoA intermediates. Unfortunately pure nafenopin- or benzafibryl-CoA were not tested for acylation of hepatocyte proteins, therefore it is not possible to distinguish between an acyl-CoA or acyl-glucuronide mechanism of covalent binding to rat liver proteins.

With long-term clinical use, clofibrate has been implicated in the formation of liver and kidney disease [2]. The covalent binding of xenobiotics to cellular macromolecules has long been associated with drug toxicity [28, 29]. By their ability to bind covalently to plasma proteins and other cellular proteins acyl-glucuronides may mediate hypersensitivity reactions, alter biliary or renal secretion by binding to transport proteins, or cause other functional changes by binding to cellular enzymes or proteins. The organs of acyl-glucuronide formation and excretion, such as liver and kidney, are potentially most at risk from the reactivity of acyl-glucuronide conjugates since they are likely to be exposed to the highest localized quantities of conjugates Many of the new lipid-lowering drugs such as gemfibrozil and the HMG-CoA reductase inhibitors are metabolized *in vivo* to carboxylic acids [30, 31], and are therefore potential substrates for acyl glucuronidation. Clearly further studies are required to elucidate the role of acyl-glucuronide conjugates in the formation of drug–protein adducts and their biological consequences.

Acknowledgements—This work was presented, in part, at the 24th Annual Scientific Meeting of the Australasian Society of Clinical and Experimental Pharmacologists, held in Melbourne, Australia, 1990.

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