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Evidence of multiple forms of rat liver microsomal coenzyme A ligase catalysing the formation of 2-arylpropionyl-coenzyme A thioesters

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Abstract—This study has demonstrated the involvement of multiple forms of rat hepatic microsomal CoA ligases in the formation of 2-arylpropionyl-CoA thioesters. In the presence of (−)*R*-ibuprofen (0.1 μM–1 mM) two enzymic processes were observed, one of which exhibited enantiospecificity and apparent high affinity for the *R* enantiomer (K_m 0.06 μM) whilst the second, a low-affinity component was non-enantiospecific. An equivalent high-affinity isoform catalysing *R*-flurbiprofen-CoA formation at concentrations less than 100 μM was not demonstrated. However, at higher substrate concentrations formation of both *R*- and *S*-flurbiprofenyl-CoA thioesters occurred. Marked inter-individual variation was observed in the formation of *S*-ibuprofen-CoA and *S*-flurbiprofen-CoA in the rats studied.

It is generally accepted that metabolic chiral inversion of the (−)*R* enantiomers of 2-arylpropionic acids (2-APAs*) such as ibuprofen and fenoprofen involves formation of a CoA thioester intermediate. In rats, this reaction is catalysed by hepatic microsomal long-chain fatty acid CoA ligase (EC 6.2.1.3) which exhibits apparent enantiospecificity for the (−)*R* enantiomers [1, 2]. Using synthetic CoA esters of both the (−)*R* and (+)*S* enantiomers of ibuprofen and flurbiprofen it has been demonstrated that the subsequent steps in this metabolic pathway (i.e. racemization and hydrolysis of the arylpropionyl-CoA intermediates are not enantiospecific [3, 4]. Collectively these data indicate that the substrate specificity and intrinsic clearance of the microsomal long-chain CoA ligase determines both the incidence and extent of *R*-2-APA-CoA thioester formation in rats. Incorporation of xenobiotic carboxylic acids into lipids proceeds via an acyl-CoA intermediate and Sallustio *et al.* [5] have demonstrated that at concentrations less than 50 μM *R*-fenoprofen is incorporated into rat hepatocyte triacylglycerols. However, at fenoprofen concentrations of 100–5000 μM a second enzymatic process was apparent which was not enantiospecific thus suggesting formation of *S*-fenoprofen-CoA. A recent study [6] has demonstrated high- and low-affinity isoforms of a rat microsomal long-chain ligase and provided evidence that formation of *R*-2-APA-CoAs cannot be fully explained by an interaction with the high-affinity isoform of a long-chain (palmitoyl) CoA ligase. The present study was undertaken to investigate further the involvement of multiple forms of microsomal long-chain fatty acid CoA ligase in the formation of 2-arylpropionyl-CoA thioesters.

Materials and Methods

The enantiomers of ibuprofen and flurbiprofen (*R* > 96% and *S* > 98% optical purity) were obtained from the Boots Co. (Nottingham, U.K.) and the CoA derivatives of the *S* enantiomers were prepared as previously described [7]. Livers (N = 8) from male outbred hooded Wistar rats (200–250 g) were used for the preparation of hepatic microsomes using a standard differential ultracentrifugation procedure. The microsomal preparations were stored in 20% glycerol, 0.1 M sodium phosphate buffer (pH 7.4) at −70° until use. Preliminary studies had established that the preparations were stable over a 3 month period. Formation of 2-APA-CoAs was assessed by quantifying the disappearance of reduced CoA (CoASH) essentially as described by Ponchaut *et al.* [8]. Optimal reaction conditions were defined in relation to protein concentration, duration of incubation and cofactor requirements. Additionally, in either the absence of protein, the presence of boiled protein or the absence of either substrate or cofactors, there was consistently less than 2% loss of CoASH. The incubation mixture (0.5 mL) contained 0.1 M sodium phosphate (pH 7.4), 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM CoASH, 0.01% Triton X-100 and either *R*-ibuprofen (0.1 μM–1 mM), *S*-ibuprofen (1 μM or 1 mM), *R*-flurbiprofen (0.1 μM–1 mM) or *S*-flurbiprofen (1 μM or 1 mM). The reaction was initiated by the addition of 100 μg of microsomal protein and incubated (37°) for 15 min. An aliquot (0.3 mL) of the incubation was then added to 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 mM sodium phosphate buffer (pH 7, 2.7 mL) and the concentration of free CoASH was calculated from the change in absorbance at 412 nm. Control incubations were performed in the absence of protein. Kinetic data (K_m and V_{max}) were calculated for *R*-ibuprofen using an extended least squares regression modelling program MKMODEL [9] from initial estimates obtained from Eadie–Hofstee plots of data from four individual rats.

Following incubation with the *S* enantiomers (1 mM) in which loss of CoASH was observed, the reaction medium was spotted onto glass TLC plates coated with silica gel containing a fluorescent indicator (Sigma Chemical Co., St Louis, MO, U.S.A.) and placed in a solvent system comprising iso-butanol/acetic acid/water (5:2:3, by vol.). The plates were allowed to develop, air dried, visualized under UV light, subsequently sprayed with 3% NaOH in methanol followed by a nitroprusside reagent [10] and the R_f values of the various solutes calculated.

Results and Discussion

Using a substrate range of 0.1 μM to 1 mM it was apparent that the rate of loss of CoASH in the presence of *R*-ibuprofen exhibited biphasic kinetics (Fig. 1A). These data suggest the presence of at least two isoforms of a microsomal CoA ligase catalysing the conjugation of CoA with *R*-ibuprofen. Large inter-individual variation in the kinetic profiles was observed and the values for the Michaelis–Menten parameters for the high-affinity, low-capacity component were K_{m_1} 0.06 ± 0.05 μM and V_{max_1} 28.2 ± 9.2 nmol/mg/min (mean ± SD, N = 4). In rats, heterogeneity of hepatic microsomal palmitoyl-CoA ligase(s) has been demonstrated and *R*-ibuprofen is a competitive inhibitor of the high-affinity component of palmitoyl-CoA formation [6]. Mutual inhibition of *R*-ibuprofen-CoA formation by palmitic acid (IC_{50} < 0.1 mM)

* Abbreviations: 2-APA, 2-arylpropionate, CoASH, coenzyme A (reduced).

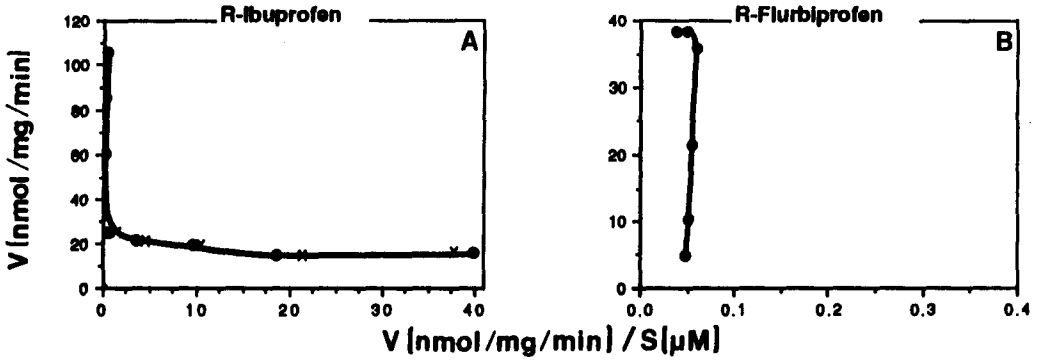

Fig. 1. Eadie–Hofstee plots of (A) *R*-ibuprofenyl and (B) *R*-flurbiprofenyl CoA ligase activity in hepatic microsomes from rat five. Ligase activity was determined by quantifying the consumption of CoASH over a substrate range of 0.1 μM to 1 mM *R*-ibuprofen and *R*-flurbiprofen. The experimentally determined data points are represented by (●) (A and B) whilst (×) denotes estimates derived using MKMODEL.

has also been reported [11] and this suggests that both compounds recognise a common catalytic site and are substrates for a microsomal long-chain ligase. Several studies using either rat liver homogenate or hepatocytes and *R*-ibuprofen [12, 13] have demonstrated chiral inversion and it is likely at the concentrations used in those studies (0.05–1 mM) that *R*-APA-CoA thioester formation would have been catalysed predominantly by the alternative high-capacity component. The data for the second enzymic process was not modelled because at the higher concentrations of *R*-ibuprofen there was evidence of atypical kinetics. These results indicate clearly that in varying the substrate concentration the rate of formation of *R*-ibuprofen-CoA will reflect the predominance of either the low- or the high-capacity isoforms.

Lack of formation of *R*-flurbiprofen-CoA has been reported *in vitro* [2, 14], whilst *in vivo* limited chiral inversion of *R*-flurbiprofen has been observed at plasma concentrations of 0.2 and 0.4 mM [15, 16]. In this study, loss of CoASH occurred only at flurbiprofen concentrations

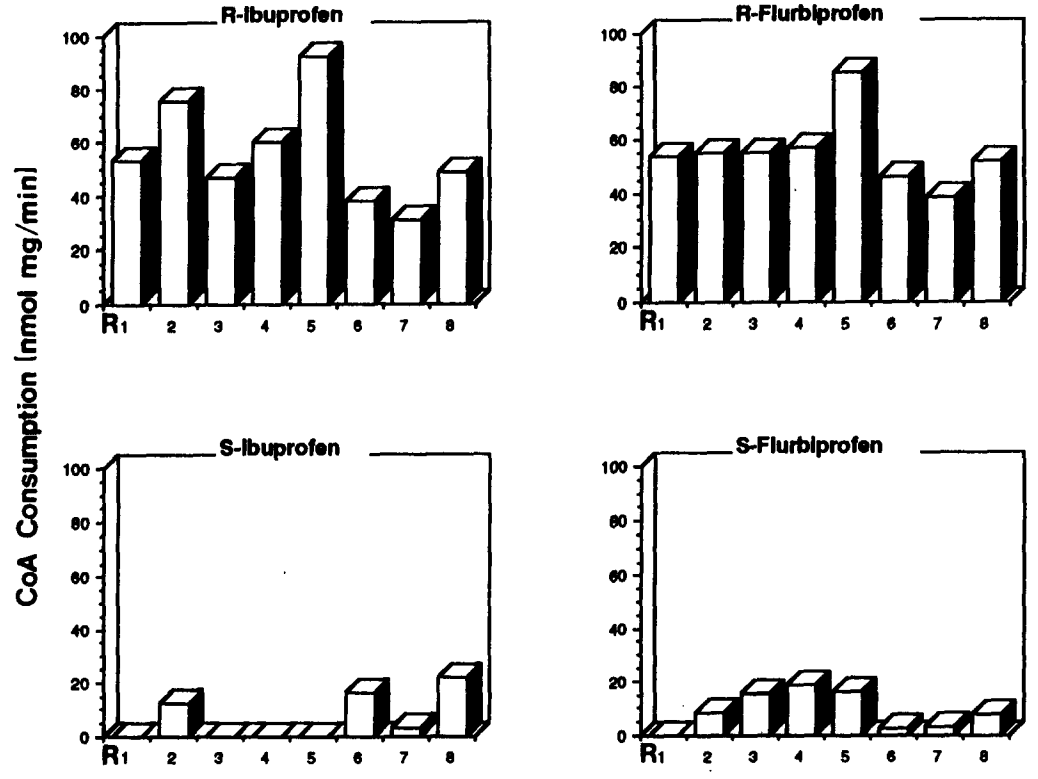

Fig. 2. CoASH consumption in the presence of either the *R* or the *S* enantiomers of ibuprofen or flurbiprofen. Hepatic microsomal protein was incubated with 1 mM substrate and the consumption of CoASH determined as described in Materials and Methods. Data are presented as individual results from eight rats.

in the range of 0.1 to 1 mM, however aberrant kinetics were consistently observed (Fig. 1B) and thus fitting of the data to Michaelis–Menten kinetics was not undertaken. At concentrations less than 100 μM there was no evidence of CoASH loss, thus an equivalent high-affinity component similar to that observed with *R*-ibuprofen was not demonstrated.

Marked consumption of CoASH was evident in the presence of both of the *R* enantiomers at the highest concentration i.e. 1 mM (Fig. 2). Of interest, however, was the observation of CoASH loss in the presence of *S*-ibuprofen and *S*-flurbiprofen. This clearly indicated that enantiospecificity was lost at the highest concentration, a situation analogous to that reported by Sallustio *et al.* [5] in relation to the enantiomers of fenoprofen. Formation of *S*-CoAs was confirmed in that analysis of the incubation medium by TLC revealed metabolites with R_f values corresponding to those of the authentic synthetic compounds (R_f = 0.35 and 0.54 for *S*-ibuprofen-CoA and *S*-flurbiprofen-CoA, respectively). In addition, the enzymically formed *S*-CoAs stained positively for a sulphydryl group using a nitroprusside reagent and when subjected to mild alkaline hydrolysis only free CoASH and the substrate were detected. Formation of *S*-ibuprofen-CoA and *S*-flurbiprofen-CoA varied 25- and 20-fold, respectively and in one rat (R1) *S*-CoA formation was not detected, while three animals (R3, 4 and 5) formed *S*-flurbiprofen-CoA only (Fig. 2). At a concentration of 1 μM, formation of either *S*-ibuprofen- or *S*-flurbiprofen-CoA was not observed in any animal and this may account for the general observation of unidirectional chiral inversion. These data suggest that measurable metabolism of *S*-flurbiprofen and *S*-ibuprofen via a CoA intermediate may occur at higher substrate concentrations (1 mM), however it is highly variable and therefore may account for the sporadic reports of chiral inversion of *S*-APAs [17].

In summary, it is apparent that conjugation of *R*-ibuprofen with CoA occurs over a wide substrate concentration range (0.1 μM–1 mM). Two enzymic processes were observed of which one exhibited an apparent high-affinity for *R*-ibuprofen and is therefore likely to be the isoform active at physiologically relevant substrate concentrations. In contrast, an equivalent high-affinity isoform involved in the formation of *R*-flurbiprofen-CoA at substrate concentrations of 0.1 to 80 μM was not observed. Increasing the substrate concentration to 1 mM resulted in loss of enantiospecificity and in contrast to the *R* enantiomers formation of *S*-ibuprofen- and *S*-flurbiprofen-CoA was highly variable. The results of this study further substantiate the pivotal role of microsomal CoA ligases in the metabolism of 2-APAs and in addition provide evidence that the choice of substrate concentration is also a crucial determinant of arylpropionyl-CoA formation. Further studies are currently investigating the apparent heterogeneity of microsomal CoA ligases.

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