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CHIRAL INVERSION OF 2-ARYLPROPIONIC ACID NON-STEROIDAL ANTI-INFLAMMATORY DRUGS—1

IN VITRO STUDIES OF IBUPROFEN AND FLURBIPROFEN

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Abstract—The mechanism of inversion of the enantiomers of 2-arylpropionic acids was investigated *in vitro* using tissue homogenates. Crude rat liver homogenate was shown to mediate the inversion of *R* to *S*-ibuprofen, but not inversion of the *S* to the *R*-enantiomer. Inversion required CoA and ATP as cofactors. In contrast, *R*-ibuprofen was not inverted by homogenates of kidney or small intestine and there was no inversion of the enantiomers of flurbiprofen by any of these tissue homogenates. Long-chain acyl-CoA synthetase was partially purified from rat liver microsomes and bound to Matrex Gel Red A. *R*-Ibuprofen was shown to be a substrate for this enzyme while *S*-ibuprofen and *R* and *S*-flurbiprofen were not substrates. These data are consistent with the hypothesis that the stereospecificity of inversion is controlled by the acyl-CoA synthetase. *R*-Ibuprofen–CoA did not racemize in either buffer solution (pH 7.4) or human plasma consistent with the hypothesis that racemization of the CoA thioesters is mediated enzymatically.

2-Arylpropionic acids (2-APAs)† are widely used in the treatment of the arthropathies because of their potent anti-inflammatory and analgesic activity. This activity, as determined by inhibition of prostaglandin synthetase, resides in the *S*-enantiomers while the *R*-enantiomers are inactive [1–7]. However, with the exception of naproxen, these drugs are used clinically as their racemates. A very interesting aspect of the disposition of members of this group of drugs is the metabolic inversion of the inactive *R*-enantiomers to the active *S*-enantiomers. Inversion appears to be stereospecific for the *R*-enantiomers and there are inter- and intra-species variability in the extent of inversion [8–15].

In abstract, Nakamura *et al.* [16] presented data based on studies of ibuprofen, which supported the schema depicted (Fig. 1) whereby; (i) *R*-ibuprofen is metabolized stereospecifically to its CoA thioester, (ii) the CoA thioester is racemized by a non-stereoselective racemase, and (iii) the *R* and *S*-CoA thioesters are cleaved by a hydrolase to release *R* and *S*-ibuprofen. In the present study, enantiomers of ibuprofen were incubated with homogenates of rat liver, kidney and small intestine to determine whether these tissues mediated inversion. Additionally the stereospecificity of acyl-CoA synthetase isolated from rat liver microsomes was investigated using the enantiomers of ibuprofen and flurbiprofen as substrates. Preliminary data on the susceptibility of *R*-ibuprofen-CoA to non-enzymatic racemization are reported. The data are further support for the mechanism of inversion proposed by Nakamura *et al.* [16].

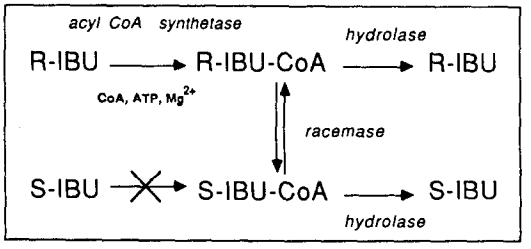

Fig. 1. Mechanism of inversion proposed by Nakamura *et al.* [16]. *R*-Ibuprofen is stereospecifically activated to *R*-ibuprofen-CoA by an acyl-CoA synthetase. *R*-Ibuprofen-CoA is racemized enzymatically to *S*-ibuprofen-CoA. The racemase (epimerase) is not enantioselective. The CoA thioesters are subsequently cleaved by an hydrolase to release *R* and *S*-ibuprofen. *S*-Ibuprofen is not a substrate for the acyl-CoA synthetase.

MATERIALS AND METHODS

General. Pure *R*(−)- and *S*(+)-enantiomers of ibuprofen and flurbiprofen were obtained from the Boots Company PLC (Nottingham, U.K.) Reduced CoA (sodium salt), ATP (disodium salt) and Triton X-100 were purchased from the Sigma Chemical Co. (Poole, U.K.). Matrex Gel Red A was purchased from the Amicon Corporation (Australia). [^{14}C]Palmitic acid was purchased from Amersham International (Amersham, U.K.). Protein concentrations were determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

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† Abbreviations used: 2-APAs, 2-arylpropionic acids; DTT, dithiothreitol; ATP, adenosine 5′-(tetrahydrogen triphosphate); AMP, adenosine 5′-(tetrahydrogen monophosphate).

Centrifugation was performed in either a Beckman cold centrifuge, using a JA 20 fixed angle rotor, or a Centrikon T-2000 ultracentrifuge using a Kontron TFT 80.13 fixed angle rotor. All buffer pH values quoted were measured at room temperature.

Animals and preparation of organ homogenates. Animal studies were approved by the Garvan Institute of Medical Research Animal House Ethics Committee. Male Wistar-strain rats (200–250 g) were purchased from the Garvan Institute (Australia). Animals were allowed free access to food and water. Following ether anaesthesia the liver was flushed with cold saline via the portal vein and excised immediately. The liver was homogenized in 5 vol. of buffer (50 mM Tris–HCl pH 7.7, 0.25 M sucrose and 50 mM $MgCl_2$) using a mechanically driven Teflon–glass homogenizer (10 vertical passes). Cellular debris was separated by centrifugation (15 min, 4°, 2000 *g*). Kidneys and small intestine were removed from an anesthetized animal, washed with cold saline, homogenized and centrifuged as for the liver preparation.

In vitro *tissue inversion studies.* The standard incubation mixture contained Tris–HCl (50 mM, pH 7.7), ATP (3 mM), CoA (0.4 mM), either *R* or *S*-ibuprofen, or *R* or *S*-flurbiprofen (50 μg/ml; 0.2 mM) and 250 μl (20 mg/ml protein) of homogenate (rat liver, kidney or small intestine) and was incubated at 37° for 30 min in a shaking water bath. Individual incubations (0.5 ml) were used for each time point.

Samples were removed from the water bath and treated in two ways:

(i) the samples were acidified (0.5 ml, 3 N HCl), extracted (10 ml hexane) and assayed for *R* and *S*-enantiomers, and

(ii) the samples were subjected to alkaline hydrolysis (100 μl, 2 N NaOH, 30 min at room temperature) followed by acidification (0.5 ml 3 N HCl) and extraction (10 ml hexane). The concentrations of drug in these samples represent the total recovery of the *R* and *S*-enantiomers. The difference between the concentrations following acidification and alkaline hydrolysis represents the amount of drug present as labile conjugates, most probably CoA thioesters.

Enantiospecific analysis of 2-arylpropionic acids. Ibuprofen and flurbiprofen enantiomers were assayed by HPLC as their diastereomeric 2-octylesters as described previously [18, 19].

Preparation of immobilised acyl-CoA synthetase. The procedure was that of Merrill *et al.* [20] with minor modifications. Six rats were anesthetized with ether, the livers were flushed with cold saline and excised immediately. The livers were minced in Tris–HCl (50 mM, pH 7.4, 150 ml), sucrose (0.25 M) EDTA (5 mM) and dithiothreitol (DTT; 5 mM) and homogenized as previously. The homogenate was centrifuged (10 min, 600 *g*) and the supernatant decanted and again centrifuged (20 min, 10,000 *g*). The resulting supernatant was centrifuged at high speed (1 hr, 105,000 *g*). The high speed pellet was resuspended by homogenization in buffer solution (100 ml) minus sucrose and centrifuged (1 hr, 105,000 *g*). This washed pellet was resuspended by homogenization in buffer solution (100 ml) minus sucrose and stirred gently while Triton X-100 (4 ml 20% w/v) was added dropwise. After 1 hr the insoluble material was removed by centrifugation (1 hr, 105,000 *g*) and the resulting clear supernatant was divided into aliquots and stored at −20°. All steps were performed at 4°. The activity of the Triton X-100 solubilized enzyme was determined using [^{14}C]-labelled palmitic acid after the method of Krisans *et al.* [21]. Matrex Gel Red A was washed with urea (6 M) in NaOH (0.5 M, 3 × 50 ml) and equilibrated with buffer solution consisting of Tris-HCl (0.1 M, pH 7.4), Triton X-100 (0.1%) and DTT (5 mM). The Triton X-100 solubilized enzyme (25 ml and approximately 100 mg of protein) was allowed to thaw at room temperature. It was added to the gel, stirred gently (4 hr, 4°) and then poured into a small chromatographic column (1 × 10 cm). The unbound material was allowed to elute from the column. Additional proteins were removed as described previously [20]. The column was stored overnight at 4°.

Enzymatic synthesis of CoA thioesters. The column was removed from the cold and allowed to equilibrate at room temperature. A reaction mixture (15 ml) containing *R* or *S*-enantiomers of either ibuprofen or flurbiprofen (250 μg/ml; 1.2 mM, 1.0 mM), CoA (2 mM), ATP (8 mM), $MgCl_2$ (30 mM), EDTA (1 mM), DTT (5 mM) and Triton X-100 (0.1%) in Tris–HCl buffer (50 mM) and a final pH of 8.2 was recycled through the column (1.0 ml/min) for 5 hr at room temperature. Aliquots were assayed for either ibuprofen or flurbiprofen enantiomers as described previously.

Racemization of R-*ibuprofen-CoA.* *R*-Ibuprofen-CoA was synthesized using the enzymatic method discussed previously. Column eluate collected after 4 hr recycling was acidified (pH 1–2), extracted with hexane (2 × 10 ml) to remove unreacted ibuprofen and then further extracted with ether (2 × 10 ml) to remove free CoA. The solution was then adjusted to neutral pH and aliquots (100 μl; 25 μg) were incubated with; (i) buffer solution (0.1 M Tris–HCl, pH 7.4, 37°; final volume 1 ml) and, (ii) fresh human plasma (final volume 1 ml) for up to 4 hr. The samples were assayed for *R* and *S*-ibuprofen, before and after mild alkaline hydrolysis (100 μl, 2 N NaOH, 30 min, room temperature).

RESULTS

Cofactor requirements and tissue selectivity for inversion

Inversion of *R* to *S*-ibuprofen, requiring ATP and CoA was observed in rat liver homogenate following incubation with *R*-ibuprofen (30 min, 37°, Fig. 2a–d). There was a commensurate decrease in the concentration of *R*-ibuprofen with formation of *S*-ibuprofen. If either cofactor was not added to the incubation, *S*-ibuprofen detected after 30 min was not significantly greater than the optical impurity in the reference *R*-enantiomer.

When *R*-ibuprofen was incubated with rat kidney or small intestine homogenate and cofactors under the same conditions as for liver homogenate no inversion was observed and the concentration of *S*-ibuprofen measured after alkaline hydrolysis was not significantly different from control values (Fig. 2e and f). Similarly, *S*-ibuprofen was not inverted, nor

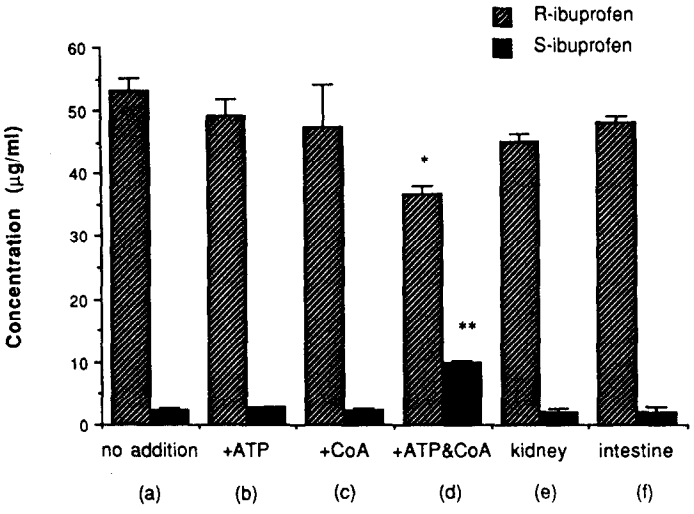

Fig. 2. Cofactor requirements and inversion of *R* to *S*-ibuprofen by rat tissue homogenates. *R*-Ibuprofen (50 μg/ml) was incubated with liver homogenate and (a) no cofactors, (b) ATP added, (c) CoA added and (d) both ATP and CoA added, for 30 min at 37°. The enantiomer concentrations shown are those determined following alkaline hydrolysis and represent the total amount of *R* and *S*-ibuprofen present in free and conjugate form. When both cofactors were added (d) there was a significant decrease in *R*-ibuprofen (*P = 0.0002) and increase in *S*-ibuprofen concentrations (**P = 0.0008), as determined using unpaired, 2-tailed *t*-tests (N = 3). *R*-Ibuprofen was incubated under the same conditions as (d), with (e) rat kidney homogenate, and (f) rat intestine homogenate. The data show that there was no inversion of *R*-ibuprofen (N = 3).

was inversion observed for either *R* or *S*-flurbiprofen when incubated with rat liver, kidney or small intestine homogenates.

Time course of inversion of R-*ibuprofen by rat liver homogenate*

When *R*-ibuprofen was incubated with rat liver homogenate which had been fortified with cofactors, there was an initial decrease in the *R*-ibuprofen concentration (Fig. 3a). There was no decrease in *R*-ibuprofen when CoA was omitted from the incubation medium (Fig. 3a) suggesting that a CoA thioester conjugate was being formed. Consequently, the initial disappearance of *R*-ibuprofen was not reflected by a similar appearance in *S*-ibuprofen. There was, however, a slow appearance of unconjugated *S*-ibuprofen. The *R*-ibuprofen concentration increased after 10 min presumably due to hydrolysis of the conjugate. It appeared that after approximately 10 min incubation there was no further formation of the conjugates and no further racemization (Fig. 3b). The early accumulation of the conjugates suggested that formation exceeded hydrolysis during this time and under these conditions.

Stereospecificity of CoA thioester formation

Partially purified acyl-CoA synthetase from rat liver microsomes exhibited high activity for palmitoyl-CoA formation. Incubation of this enzyme preparation with [^{14}C]palmitic acid resulted in 95.5% converstion to [^{14}C]palmitoyl-CoA in 9 min. Application of partially purified acyl-CoA synthetase (100 mg of protein) to Matrex Gel Red A resulted in the retention of approximately 10 mg protein/ml of gel. Acyl-CoA synthetase catalysed the formation of *R*-ibuprofen-CoA (Fig. 4) as measured by the disappearance of *R*-ibuprofen from the circulating medium. The reaction was complete in approximately 4 hr. Following mild alkaline hydrolysis, 92% of total ibuprofen was recovered as *R*-ibuprofen and 8% as *S*-ibuprofen (Fig. 4). When CoA was excluded from the reaction medium there was no formation of the conjugate, *R*-ibuprofen-CoA (Fig. 4). Acyl-CoA synthetase did not catalyse the formation of either *S*-ibuprofen-CoA or *R* or *S*-flurbiprofen-CoA.

No spontaneous racemization of R-*ibuprofen-CoA*

Buffer. *R*-Ibuprofen-CoA did not racemize when incubated at 37° for up to 4 hr in buffer solution (Fig. 5a). Following mild alkaline hydrolysis, 92% of *R*-ibuprofen-CoA was recovered as *R*-ibuprofen. A small amount of *S*-ibuprofen (8%) was evident in all samples including the zero time sample. However, there was no further increase in the *S*-ibuprofen concentration over the 4 hr incubation. Assay of these samples prior tc alkaline hydrolysis indicated that *R*-ibuprofen-CoA once formed, was stable under these conditions.

Plasma. *R*-Ibuprofen-CoA did not racemize when incubated with human plasma at 37° for up to 4 hr (Fig. 5b). However, in contrast to the buffer medium, there was gradual hydrolysis of the *R*-ibuprofen-CoA thioester (32% at 4 hr). Following alkaline hydrolysis 92.7% of ibuprofen was recovered in the *R*-form and the remainder in the *S* form.

DISCUSSION

The requirement for CoA and ATP for the stereospecific inversion of *R*-ibuprofen to *S*-ibuprofen by rat liver homogenate (Fig. 2) confirmed the previous

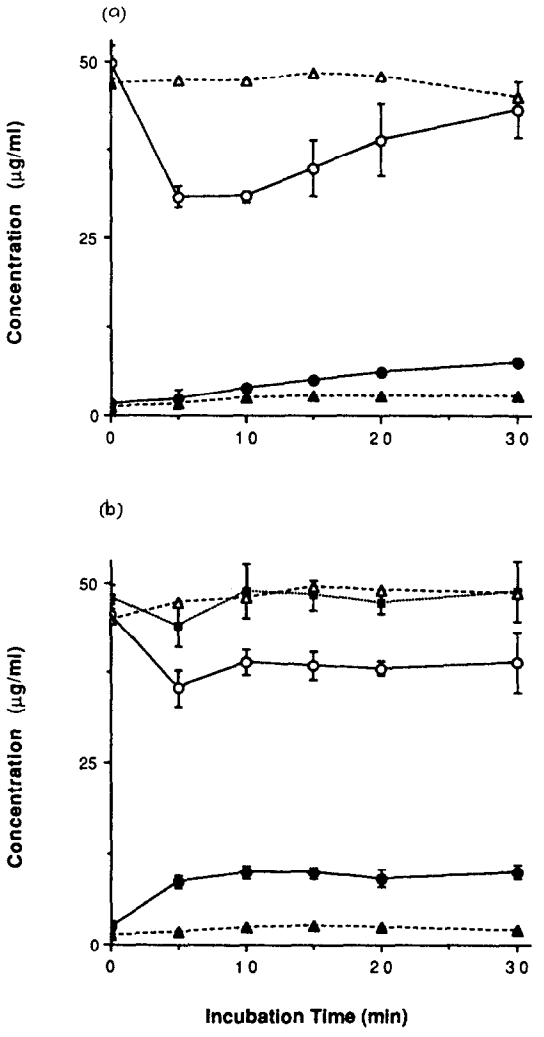

Fig. 3. Time course of ibuprofen enantiomer concentrations following incubation of *R*-ibuprofen (50 μg/ml) with rat liver homogenate (30 min, 37°). (a) *R*-Ibuprofen (○—○) and *S*-ibuprofen (●—●) concentrations (N = 3) prior to alkaline hydrolysis. *R*-Ibuprofen (△---△) and *S*-ibuprofen (▲---▲) concentrations when no CoA was added to the incubation (N = 1). (b) *R*-ibuprofen (○—○) and *S*-ibuprofen (●—●) enantiomer concentrations following alkaline hydrolysis (N = 3). *R*-Ibuprofen (△---△) and *S*-ibuprofen (▲---▲) concentrations when no CoA was added to the incubation (N = 1). Sum of *R* and *S*-ibuprofen (■---■) showing no change with time in the total concentration of ibuprofen.

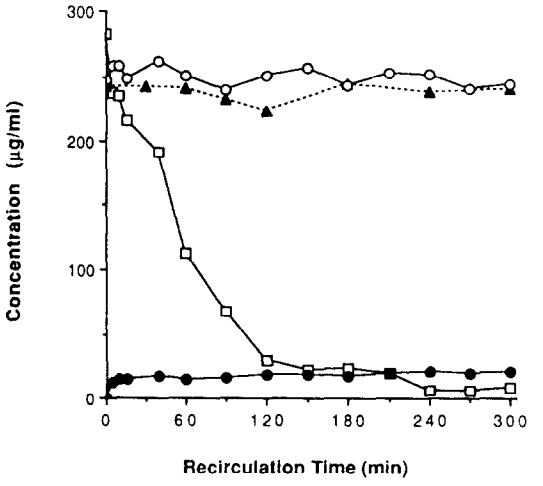

Fig. 4. Formation of *R*-ibuprofen-CoA thioester by rat liver microsomal acyl-CoA synthetase. The data show the disappearance of *R*-ibuprofen (□—□) from the reaction medium following recirculation through the column containing immobilized acyl-CoA synthetase. Reaction mixtures (15 ml) contained either *R* or *S*-ibuprofen (250 μg/ml; 1.2 mM), 8 mM ATP, 2 mM CoA, 30 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and 0.1% Triton X-100 in 50 mM Tris–HCl at a final pH of 8.2. Following mild alkaline hydrolysis, all of the *R*-ibuprofen was recovered (○—○) with a small amount of *S*-ibuprofen (●—●) present formed by racemization during alkaline hydrolysis. Also illustrated are the concentrations of *R*-ibuprofen (▲---▲) when CoA was excluded from the reaction medium.

report by Nakamura *et al.* [16]. The concentration–time profiles following incubation of *R*-ibuprofen with rat liver homogenate (Fig. 3) indicated that *R*-ibuprofen was removed from the incubation via formation of an *R*-ibuprofen conjugate. This conjugate was not isolated and characterized. However, there was no change in the *R*-ibuprofen concentration if CoA was omitted from the incubation medium (Fig. 3a) and these conjugates were rapidly hydrolysed under very mild alkaline conditions with recovery of ibuprofen (Fig. 3b). We conclude from these observations that the conjugates were the CoA thioesters of ibuprofen. No further *S*-ibuprofen was formed after 10 min incubation (Fig. 3b) indicating that the *in vitro* system was constrained, presumably by either the accumulated reaction products (CoA thioesters, AMP, pyrophosphate) having an inhibitory effect on the acyl-CoA synthetase, or by an insufficient supply of cofactors.

Our rat liver homogenate contained all enzymes necessary for inversion although other authors [22] failed to obtain inversion under similar conditions. The reason for this is unclear. In contrast to our data for liver, homogenates of kidney and small intestine did not mediate inversion. The disruption of cellular boundaries by homogenization may significantly alter the ordered sequence of events which occur *in vivo*. *In vitro*, the enzymes are able to compete directly with each other for the substrates in a non-ordered manner, for example CoA formation and CoA hydrolysis. In addition, enzyme activities in kidneys and small intestine may be low and their specificities may differ from those found in liver. The negative findings for inversion of *R*-ibuprofen by kidney and small intestine homogenates in our studies may be the result of factors such as these and as such it cannot be concluded that kidney and small intestine do not invert *R*-ibuprofen *in vivo*. In fact, Nakamura and Yamaguchi [23] have reported that 2-phenylpropionic acid was inverted by rat liver and kidney slices but not by a rat liver homogenate preparation.

Evidence so far presented suggests, in agreement with the proposed schema (Fig. 1), that an acyl-CoA synthetase is involved in the inversion of 2-APAs. In further support of this conclusion was the data obtained from the more detailed study of acyl-CoA

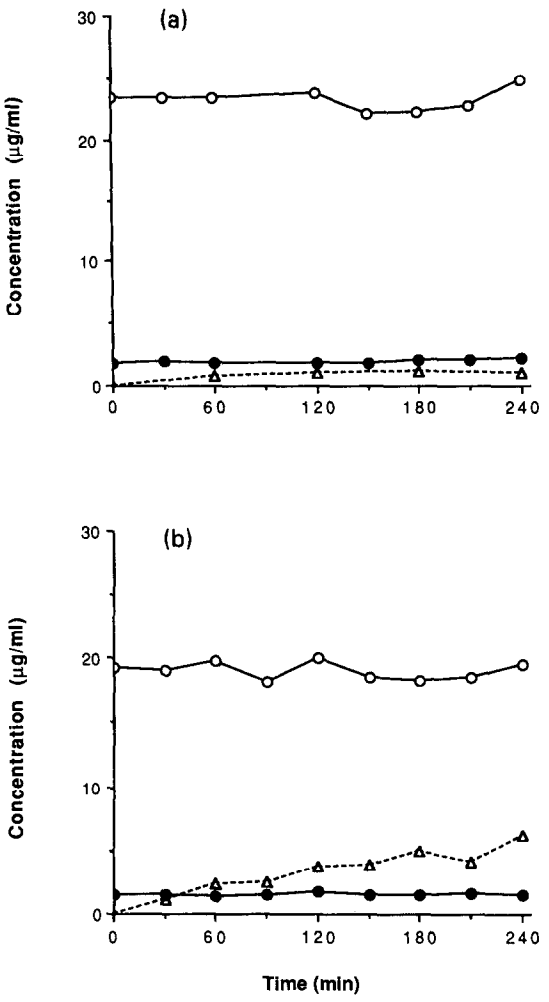

Fig. 5. Determination of *R*-ibuprofen-CoA racemization following incubation of enzymatically synthesised crude *R*-ibuprofen-CoA thioester (25 μg/ml) with, (a) buffer (Tris–HCl, 50 mM pH 7.4) and (b) fresh human plasma. (a) *R*-Ibuprofen (○—○) and *S*-ibuprofen (●—●) concentrations following mild alkaline hydrolysis and *R*-ibuprofen concentrations (△---△) prior to alkaline hydrolysis indicating the stability of the *R*-ibuprofen-CoA thioester in the respective incubation medium.

synthetase. Fatty acid competition studies have indicated the presence of only one acyl-CoA synthetase (long-chain Acyl-CoA synthetase) in rat liver microsomes [24]. This enzyme has also been shown to activate medium-chain fatty acids [25, 26]. No specific aryl carboxylic acid CoA synthetase has been found but two mammalian mitochondrial enzymes activate benzoates to their CoA thioesters [27, 28]. Considering the range of substrates that acyl-CoA synthetases are able to activate it is of interest that long-chain acyl-CoA synthetase was shown to be stereospecific for *R*-ibuprofen. Our approach was that used by Knights *et al.* [29] where *R*-fenoprofen was similarly shown to be a substrate for acyl-CoA synthetase from rat liver microsomes. The failure of liver homogenate to invert flurbiprofen was consistent with the finding that, in contrast to ibuprofen, the flurbiprofen enantiomers were not substrates for acyl-CoA synthetase. Recent studies have indicated that *R*-flurbiprofen is not inverted by rats, humans [30] or rabbits [19] but there may be inversion in guinea-pigs [2].

Other indirect evidence in support of the key role of CoA thioester formation in the mechanism of inversion is our previous data on the uptake of ibuprofen into adipose tissue in rats treated with the enantiomers of ibuprofen [31]. Administered *S*-ibuprofen was not significantly incorporated into adipose tissue consistent with its inability to form CoA thioesters. Similarly, synthesis of fenoprofen triacylglycerol by rat liver hepatocytes [32] *in vitro* was also stereospecific for the *R*-enantiomer. Thus, these data support the hypothesis that CoA formation controls the extent and stereospecificity of inversion of 2-APAs.

It has been suggested that the racemization of *R*-ibuprofen-CoA, the second step in the proposed mechanism of inversion (Fig. 1), occurs spontaneously [33] because CoA thioester formation increases the lability of the alpha-hydrogen. Data for DL-methylmalonyl-CoA [34] indicated that spontaneous tritium exchange, a measure of the rate of racemization, of only <0.05, 0.5 and 0.9% per hr was observed at 4, 30 and 37°, respectively. Furthermore, Mayer *et al.* [22] observed that a model thioester of phenylpropionic acid (2-dimethylamino-ethanethiol 2-phenylpropionate) did not racemize in aqueous or methanol/water solutions but did racemize in a less polar (acetonitrile/water) environment. However, we found no racemization of *R*-ibuprofen-CoA in buffer over the 4 hr incubation period. The *S*-ibuprofen present at zero time (8%) was the result of racemization during alkaline hydrolysis. The *R*-ibuprofen-CoA used for this study was not isolated and purified. However, we believe it unlikely that the presence of detergent or cofactors from the column preparation would have significantly impeded or enhanced racemization. We also found that *R*-ibuprofen-CoA did not racemize when the crude preparation was incubated with fresh plasma. In contrast with the buffer medium, there was gradual hydrolysis of the thioester, presumably the result of enzymatic hydrolysis or instability of the thioester in plasma. Our results are preliminary evidence that racemization of *R*-ibuprofen-CoA is mediated by an enzyme.

We have not characterized the hydrolase(s) involved in the cleavage of the CoA thioesters, although it is clearly present in the liver homogenate. There are a number of potential candidates for this function. It is likely that the enzyme will belong to one of the fatty acid thioesterases. Some of these are relatively non-selective such as palmitoyl-CoA hydrolase [35] while others such as *S*-methylmalonyl-CoA hydrolase [36] are highly enantioselective. The data for this latter enzyme suggest that there may even be two distinct enzymes responsible for hydrolysis of each of the CoA thioesters of *R*-ibuprofen and *S*-ibuprofen.

The mechanism of inversion of 2-APAs is a biotransformation so far unique to this group of drugs. Future studies will be directed at further characterizing the enzymes responsible for this novel transformation. The study of this system has broader implications for the potential toxicities of the thio-

esters of the arylpropionates and the relationship between the disposition of this class of drug and the endogenous enzymes responsible for their transformation.

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