Biochemical Pharmacology, Vol. 44, No. 7, pp. 1468–1470, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Influence of palmitate and benzoate on the unidirectional chiral inversion of ibuprofen in isolated rat hepatocytes

(*Received* 3 *April* 1992; *accepted* 15 *July* 1992)

Abstract—The influence of benzoic acid, a typical substrate of medium-chain acyl-CoA synthetase, and of palmitic acid, a substrate of long-chain acyl-CoA synthetase, on the metabolic chiral inversion of ibuprofen was investigated in freshly isolated hepatocytes. It was shown that the conjugation of benzoid to hippuric acid does not influence the chiral inversion of ibuprofen. In contrast, palmitic acid inhibited markedly the *R*-to-*S* inversion of ibuprofen. It was concluded that this inhibition is due to competition between (*R*)-ibuprofen and palmitic acid for long-chain acyl-CoA synthetases.

Ibuprofen, a non-steroidal anti-inflammatory drug, is used therapeutically as the racemate. Its (−)-(*R*)-enantiomer was shown to undergo substantial inversion of configuration at the asymmetric centre (C-2 position) [1, 2], the proposed mechanism involving the stereoselective formation of a CoA acyl-thioester with subsequent racemization and hydrolysis [3]. Several *in vitro* studies have confirmed that only the (*R*)-enantiomer of ibuprofen and other 2-arylpropionates can yield acyl-CoA thioesters [4, 5]. Of potential toxicological significance is the fact that the acyl-CoA may be pivotal not only in the chiral inversion process, but also in the formation of hybrid triglycerides [6–8] and even in the inhibition of fatty acid β-oxidation [9].

The present study investigates the influence of benzoic acid, a typical substrate of medium-chain acyl-CoA synthetases (EC 6.2.1.2), and palmitic acid, a substrate of long-chain acyl-CoA synthetases (EC 6.2.1.3), on the chiral inversion of ibuprofen. The work was carried out in suspensions of freshly isolated rat hepatocytes, an appropriate *in vitro* model for investigating the metabolism and chiral inversion of 2-arylpropionates [10, 11].

Materials and Methods

Materials. Ibuprofen and its (−)-(*R*)- and (+)-(*S*)-enantiomers were generously donated by the Boots Co. (Nottingham, U.K.). The enantiomeric purity of each enantiomer was greater than 98%. Salts used for the preparation of Hanks' buffer were of analytical grade (Fluka, Buchs, Switzerland; Siegfried, Zofingen, Switzerland). The reagents employed for the isolation of hepatocytes, EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid] and collagenase from *Clostridium histolyticum* type IV, were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Three different types of albumin were used, bovine serum albumin (BSA) (Fluka), essentially fatty acid-free BSA and BSA–palmitic acid (1:4 molar ratio) (Sigma). Benzoic acid (pharmaceutical purity) was obtained from Siegfried, and glycine from Merck AG (Zürich, Switzerland).

The derivatization reagents *N*-(dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride and 1-hydroxy-benzotriazol were purchased from Fluka, (−)-(*S*)-α-(naphthyl)ethylamine (enantiomeric purity greater than 99%) from Sigma, 1-naphthylacetic acid from Aldrich-Europe (Beerse, Belgium), and the chromatographic solvents (HPLC grade) from Fluka. Male Sprague–Dawley rats (200–300 g) were obtained from Madörin AG (Fullinsdorf, Switzerland).

Hepatocyte isolation. Rat hepatocytes were isolated by a two-step collagenase perfusion of the liver [12] as reported previously. The cells were resuspended in Hanks' buffer [13] with 0.5% BSA containing various ratios of palmitic acid and incubated at 37° while shaking in an air/CO_2 95%/5% atmosphere. Cell viability, determined by Trypan blue exclusion, was routinely greater than 85%.

Analysis. At different time points samples were withdrawn from the suspension, and the cells separated by centrifugation. The internal standard (1-naphthylacetic acid: 125 μg/5 mL sample) was added to the supernatant. The extraction and analysis of ibuprofen were performed as described [14]. Briefly, the extracted enantiomers of ibuprofen were derivatized with (−)-(*S*)-1-naphthylethylamine to form the diastereomeric amides. Samples of 20 μL were analysed by HPLC. Benzoic and hippuric acid were analysed simultaneously by HPLC using the following conditions: detection wavelength 230 nm, LiChrosorb RP18 (10 μm) column (250 × 3 mm i.d.), mobile phase methanol-HCl pH 1.5 (5.5:4.5; v/v), flow rate 2 mL/min.

Pharmacokinetic calculations. A compartmental model was used [10], with k_{12} designated as the first-order rate constant of *R*-to-*S* conversion, and k_{10} and k_{20} designated as the first-order rate constants of metabolic elimination of (*R*)- and (*S*)-ibuprofen, respectively, by routes other than chiral inversion. Data analysis was carried out with a non-linear regression program (Siphar, Simed S.A., Créteil, France).

Results

Effect of benzoic acid. rac-Ibuprofen (48 μM) (Table 1) or (−)-(*R*)-ibuprofen (24 μM) (results not shown) were incubated with hepatocytes in the presence of benzoic acid (102 μM) and glycine (125 μM). After 3 hr, about 40% of benzoic acid was transformed to hippuric acid. During the same period about 60% of (−)-(*R*)-ibuprofen was metabolized. Thus, an excess of benzoic acid was present for the duration of the incubation. The presence of benzoic acid did not influence the metabolism of ibuprofen. Indeed, superimposable concentration–time profiles were obtained for ibuprofen enantiomers in the absence and presence of benzoic acid in the incubation medium. The calculation of the rate constants with the full kinetic model yielded a k_{10} value that did not differ significantly from zero, as observed previously [10]. This may indicate that under the conditions of study (*R*)-ibuprofen was metabolized via the inversion pathway only. The rate constants reported in Table 1 were therefore calculated with a reduced model (i.e. $k_{10} = 0$), confirming that the conjugation of benzoic to hippuric acid does not influence the chiral inversion of ibuprofen.

Effect of palmitic acid. Figure 1 shows the influence of palmitic acid on the metabolism of *rac*-ibuprofen. The concentration–time profile obtained with essentially fatty acid-free BSA shows that (*R*)-ibuprofen is more rapidly eliminated than its antipode. In comparison, no significant differences between the apparent half-lives of ibuprofen enantiomers were detectable when the incubation medium contained BSA–palmitic acid (1:4 molar ratio). Modelling

Table 1. Influence of benzoate or palmitate on the metabolism of *rac*-ibuprofen in isolated rat hepatocytes

	k_{12} (min^{-1})*	k_{20} (min^{-1})†
Control	0.0037 ± 0.0006	0.0028 ± 0.0007
Benzoic acid‡	0.0038 ± 0.0001	0.0033 ± 0.0003
Control 1§	0.0025 ± 0.0005	0.0025 ± 0.0010
Control 2‖	0.0028 ± 0.0003	0.0028 ± 0.0003
BSA–palmitic acid¶	0.0019 ± 0.0002	0.0029 ± 0.0004
BSA–palmitic acid**	0.0007 ± 0.0004	0.0013 ± 0.0003

Hepatocytes (3×10^5 cells/mL, N × 3–5) were in Hanks' buffer containing 0.5% (BSA) and *rac*-ibuprofen (48 μM). The rate constants were calculated with a reduced kinetic model ($k_{10} = 0$).

* Rate constant of unidirectional *R*-to-*S* chiral inversion (mean ± SD).

† Rate constant of elimination of (*S*)-ibuprofen (mean ± SD).

‡ Benzoic acid (102 μM), glycine (125 μM).

§ Normal BSA.

‖ Essentially fatty acid-free BSA.

¶ Molar ratio 1:2; 73.5:147 μM.

** Molar ratio 1:4; 73.5:294 μM.

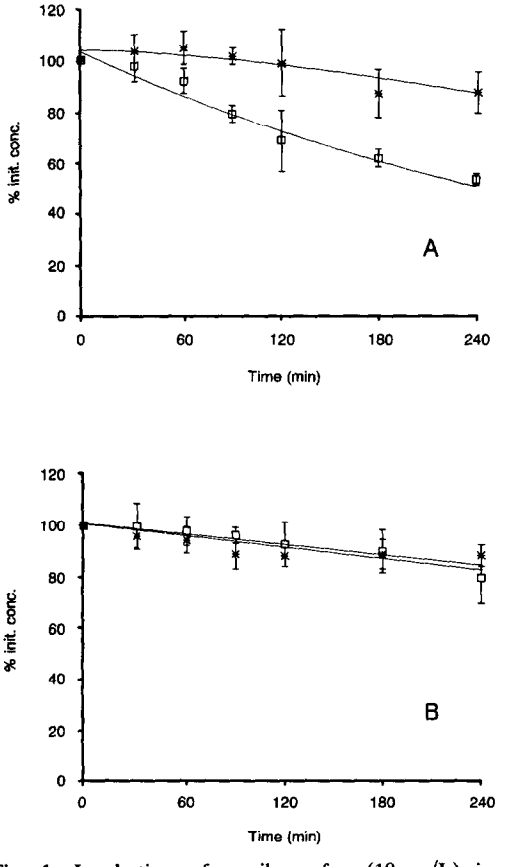

Fig. 1. Incubation of *rac*-ibuprofen (10 mg/L) in rat hepatocyte suspensions (2×10^5 cells/mL). (A) 0.5% essentially fatty acid-free BSA. (B) 0.5% BSA–palmitic acid (1:4 molar ratio). (□) (−)-(*R*)-Ibuprofen; (*) (+)-(*S*)-ibuprofen. Each point represents the mean of five experiments.

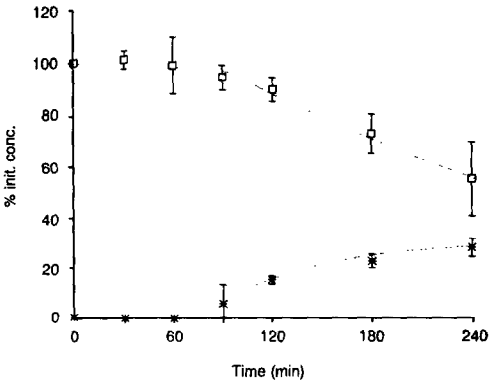

Fig. 2. Incubation of (−)-(*R*)-ibuprofen (5 mg/L) in rat hepatocyte suspensions (2×10^5 cells/mL) in an incubation medium containing 0.5% BSA–palmitic acid (1:4 molar ratio). (□) (−)-(*R*)-Ibuprofen; (*) (+)-(*S*)-ibuprofen. Each point represents the mean of three experiments.

revealed that palmitic acid decreases significantly the rate constant of *R*-to-*S* conversion (k_{12}), but has little effect on the elimination of (*S*)-ibuprofen; k_{10} was again not different from zero (Table 1).

When (−)-(*R*)-ibuprofen was incubated separately with BSA–palmitic acid (1:4 molar ratio), its concentration remained constant and no formation of its *S*-isomer was observed for about 80 min (Fig. 2). Thereafter, the *R*-enantiomer concentration decreased slowly and in parallel with the formation of its antipode. This observation seems to indicate that palmitic acid inhibited the inversion of (*R*)-ibuprofen as long as it was present in sufficiently high concentrations, and that this effect was limited by the catabolism of the fatty acid. The stereoselectivity of the action of palmitic acid was confirmed by the separate incubations of (+)-(*S*)-ibuprofen whose elimination was not affected by palmitic acid (results not shown).

Discussion

Glycine conjugation implies the activation of the target carboxylic acid to a reactive acyl-CoA intermediate, a reaction catalysed by medium-chain acyl-CoA synthetases (EC 6.2.1.2) located in the mitochondrial matrix [15]. Our observation that glycine conjugation of benzoic acid is without effect on the chiral inversion of (*R*)-ibuprofen suggests that (*R*)-ibuprofen is not a substrate for medium-chain acyl-CoA synthetases and that the levels of mitochondrial CoA are not decreased sufficiently to impair chiral inversion.

Palmitic acid and ibuprofen may interact at different levels. Thus, both acids could compete for the same binding site on BSA. A displacement of ibuprofen by palmitic acid would increase the free concentration of the xenobiotic. Since ibuprofen is a low clearance drug this displacement should result in a faster elimination [16], in contrast to our observations. It is also conceivable that the unbound fraction of ibuprofen is decreased by cooperative binding in the presence of palmitic acid. An enhancement of site I binding by long-chain fatty acids is well documented [17]. However, this effect has not been observed at the binding site II with which ibuprofen interacts [18].

It can therefore be concluded that the effect of palmitic acid on the elimination of ibuprofen does not result from interactions with albumin but is probably due to competition for long-chain fatty acid CoA synthetases. This view is supported by studies with long-chain acyl-CoA synthetase from rat liver microsomes which showed (*R*)-ibuprofen [4] and (*R*)-fenoprofen [19] to be conjugated to their respective

acyl-CoA thioesters. Recently, the effect of (*R*)-ibuprofen on the metabolism of palmitic acid was investigated in mouse liver microsomes as a function of CoA concentrations. It was found that at the concentration of CoA present in liver cell cytosol, both enantiomers slightly inhibited the β-oxidation of palmitic acid and markedly inhibited the β-oxidation of octanoic acid and butyric acid [20]. *In vivo* both enantiomers similarly increased hepatic triglycerides and both produced mild microvesicular steatosis of the liver. The present study shows that while (*R*)-ibuprofen can affect the metabolism of palmitic acid, the reverse metabolic interaction also occurs, the *R*-to-*S* inversion of ibuprofen being inhibited by palmitic acid.

Acknowledgements—Support by the Swiss National Science Foundation (grant No 31-25715.88) is gratefully acknowledged.

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