Pergamon

Biochemical Pharmacology, Vol. 48, No. 5, pp. 1056–1058, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00218-5

Is the formation of *R*-ibuprofenyl-adenylate the first stereoselective step of chiral inversion?

(*Received* 27 *December* 1993; *accepted* 4 *May* 1994)

Abstract—Coenzyme A thioester formation is reported to be the first step of chiral inversion of *R*-ibuprofen. In order to investigate the mechanism of this reaction adenylate derivatives of the ibuprofen enantiomers were synthesized chemically. *R*- and *S*-ibuprofenyl-adenylates as well as free acids were incubated with rat liver mitochondria in the presence of coenzyme A, $MgCl_2$ with or without ATP. The optical antipodes formed by inversion and the coenzyme A thioester derivatives of both enantiomers were found after incubation of both *R*- or *S*-ibuprofenyl-adenlyate and *R*-ibuprofen. By contrast, after incubation with *S*-ibuprofen neither *R*-enantiomer nor coenzyme A thioesters were detected. These experiments suggest that the formation of *R*-ibuprofenyl-adenylate may be the first stereoselective step of chiral inversion.

Key words: 2-arylpropionic acids; stereoselectivity; mechanism of inversion; intermediates; acyl adenylates; coenzyme A thioester formation

IBU* and other 2-APAs exhibit metabolic chiral inversion in a species and substrate-dependent manner *in vivo* [1]. Formation of a coenzyme A thioester with *R*-enantiomers in the presence of CoA, ATP and Mg^{2+} is reported to be the stereoselective and decisive step of inversion, followed by epimerization and hydrolysis [2–5]. The present study examined the role of ATP in the activation of R-IBU to CoA thioesters. In 1956 Berg [6] demonstrated that acetate is activated to the CoA thioester via an acyl-AMP intermediate. Recently, 2-propylpentanoic acid (valproic acid) was shown to be converted to 2-propylpentanoic-AMP in rat liver mitochondria [7]. The aim of the present investigations was, therefore, to examine whether R-IBU-AMP is formed prior to thioesterification of R-IBU and may thus be the step which determines the stereoselectivity of inversion. In order to investigate this hypothesis, the adenylates of R- and S-IBU were synthesized chemically and incubated with rat liver mitochondria. IBU enantiomers and the respective CoA thioesters in the mitochondrial suspensions were analysed stereoselectively.

Materials and Methods

IBU enantiomers were kindly supplied by Pharma Trans Sanaq AG (Basel, Switzerland). CoA, ATP and all standard chemicals were obtained from Sigma (Deisenhofen, Germany). R- and S-IBU-AMP were synthesized as described by Berg for amino acids [8].

The structures of both R- and S-IBU-AMP were demonstrated by their ^1H- and ^{13}C-NMR spectra (measured at 360 and 90 MHz, respectively, in CD_3OD-D_2O 4:1). For S-IBU-AMP the following ^{13}C-NMR shifts and ^{13}C–^{31}P couplings were measured: 172.8 (d,^2J C-P = 10.5 Hz, —O—C=O) 157.0 (C-6 AMP), 153.7 (C-2 AMP), 150.6 (C-4 AMP), 141.7 (C-1 IBU), 141.0 (C-8 AMP), 138.6 (C-4 IBU), 130.3 (C-3 IBU and C-5 IBU), 128.4 (C-2 IBU and C-6 IBU), 120.0 (C-5 AMP), 88.6 (C-1′AMP), 85.2 (d,^3J C-P = 9 Hz, C-4′AMP), 75.9 (C-2′AMP), 71.9 (C-3′AMP), 66.8 (d, ^2J C-P = 6 Hz, C-5′AMP), 47.3 (d,^3J C-P = 6 Hz, C-α IBU), 45.7 (C-β′IBU), 31.2 (C-α′ IBU), 22.6 (C-γ′ IBU) and 18.7 (C-β IBU). Very similar values for chemical shifts and couplings were found for R-IBU-AMP.

Prior to the experiments the product obtained was dissolved in cold Tris–HCl buffer (50 mM) and filtered to remove the insoluble dicyclohexylurea formed during synthesis. The adenylates were directly monitored by reversed phase HPLC as published for valproyl-AMP with the slight modification that the mobile phase consisted of 50 mM ammonium phosphate buffer pH 5.5 (65% v/v) and acetonitrile (35% v/v) at a flow rate of 1 mL/min [7]. Quantification of the adenylates was rendered possible by measuring R- and S-IBU after alkaline hydrolysis using a stereoselective HPLC method [9]. R- and S-IBU CoA thioesters were prepared as described previously [10]. Freshly-prepared liver mitochondria from adult male Sprague–Dawley rats (350 g) were obtained by differential centrifugation of liver homogenates according to standard procedures. The incubation mixtures (400 μL) consisted of Tris–HCl buffer (50 mM) pH 7.4, supplemented with KCl (150 mM), $MgCl_2$ (15 mM), substrate (either R- or S-IBU, or R-IBU-AMP or S-IBU-AMP, 0.25 mM), CoA (0.3 mM) and mitochondrial protein (1 mg). In the case of the free acids, ATP (3 mM) was also added.

Control incubations were performed in the absence of mitochondria. As a further control *R*-ketoprofen was added to the mitochondrial incubations with R-IBU-AMP. Aliquots of the incubation mixture were removed from the water bath together with controls at 1, 3, 5, 10, 30 and 60 min and assayed for R- and S-IBU following alkaline hydrolysis (pH 12, 1 hr at room temperature) [9]. Furthermore the respective thioesters of R- and S-IBU were analysed by a stereoselective HPLC method [11].

Results and Discussion

Consistent with published data [12] R-IBU was inverted in rat liver mitochondria in the presence of CoA, ATP, $MgCl_2$ and KCl as shown in Fig. 1A. Furthermore the CoA thioesters of R-IBU and S-IBU were also detected. By contrast, the *S*-enantiomer was not inverted under the same conditions and neither were thioesters formed. Following incubation of R-IBU-AMP without ATP the optical antipode was formed and CoA thioester intermediates quantified (Fig. 1B). By contrast with the free acid, S-IBU-AMP was inverted to the *R*-enantiomer when incubated under the same conditions as for R-IBU-AMP as shown in Fig. 1C. The CoA thioesters of both R- and S-IBU were detected in the supernatant mitochondrial suspension, suggesting that adenylate formation prior to

* Abbreviations: IBU, ibuprofen; 2-APAs, 2-arylpropionic acids; R-IBU-AMP, *R*-ibuprofenyl-adenylate; S-IBU-AMP, *S*-ibuprofenyl-adenylate; CoA, coenzyme A.

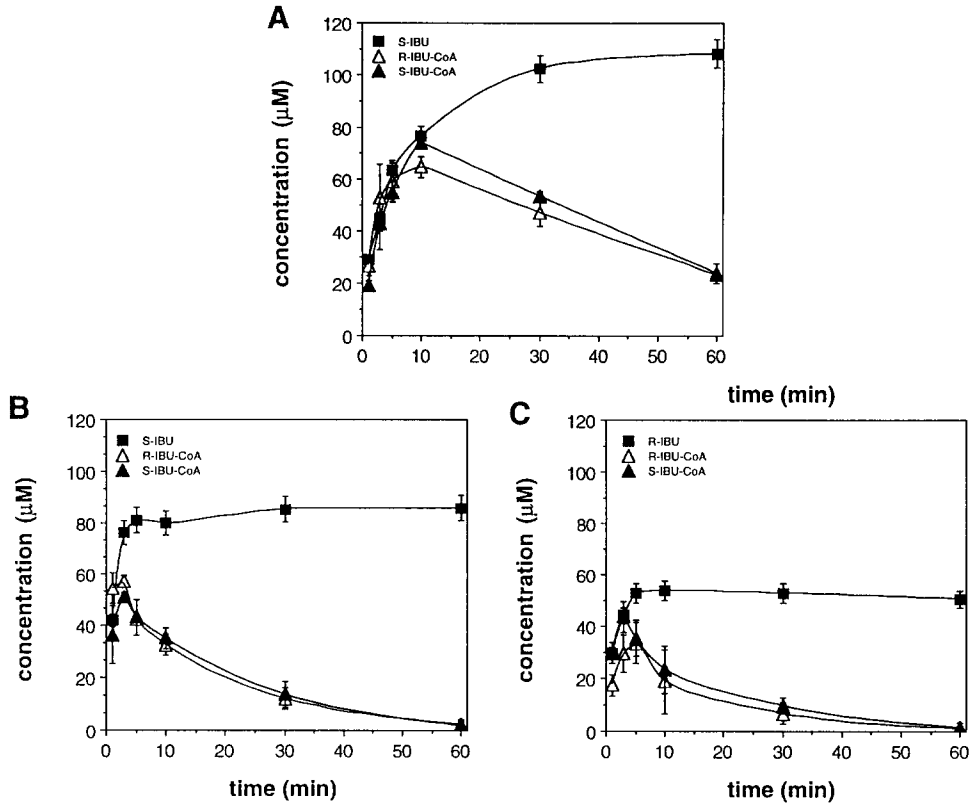

Fig. 1. Concentration–time curves of the optical antipodes formed by inversion (after alkaline hydrolysis) and of R- and S-IBU-CoA thioesters after incubation of rat liver mitochondria with R-IBU in the presence of CoA and ATP (A), and R-IBU-AMP (B) and S-IBU-AMP (C) with CoA but without ATP, respectively; (means ± SD, N = 3).

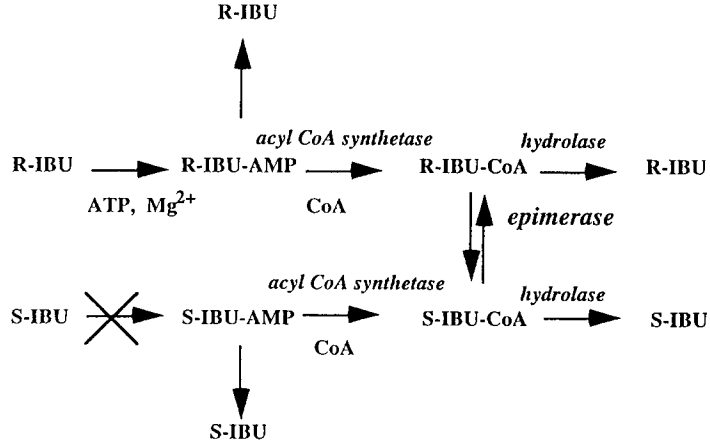

Fig. 2. Proposed scheme for extending the inversion mechanism suggested by Nakamura *et al.* [2]. R-IBU is enzymatically activated to R-IBU-AMP followed by CoA thioester formation by an acyl CoA synthetase. The thioesters are substrates for the epimerase. S-IBU is not activated to the adenylate derivative and thus not able to form a CoA thioester. Chemically synthesized S-IBU-adenylate, however, is a substrate for the acyl-CoA synthetase and able to form the thioester which is epimerized to the *R*-enantiomer.

thioesterification is the first and stereoselective step of R-IBU inversion. The somewhat lesser extent of thioester formation and inversion after incubation of R-IBU-AMP and S-IBU-AMP as compared to R-IBU may be caused by rapid hydrolysis of the acyl adenylates and/or by the unchanged adenosine 5'-monophosphate from synthesis, expected to inhibit thioester formation. No inversion was evident in control incubations without mitochondria. In addition, no inversion of *R*-ketoprofen (another 2-APA derivative inverted in rat liver mitochondria in the presence of ATP) occurred in control samples when incubated together with R-IBU-AMP, suggesting that the non-hydrolysed adenylate may be necessary for thio-esterification.

The results of these experiments lend support to the hypothesis that CoA thioester formation in itself is not the step determining the stereoselectivity of inversion of R-IBU [3, 4, 13], rather, it is the formation of an adenylate prior to thioesterification. The reaction may be mediated by long chain acyl CoA synthetases [14], forming an enzyme bound AMP-derivative as described by Berg for acetate activation [6]. Consequently, the mechanism of inversion suggested by Nakamura *et al.* [2] may be extended as presented in Fig. 2. However, IBU-AMP derivatives could not be detected in the samples probably due to rapid thioesterification and hydrolysis. Even after incubation of the IBU-AMP derivatives the adenylates could not be found with the HPLC method used.

Acknowledgement—This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (SFB 353).

Departments of †Experimental and Clinical Pharmacology and of ‡Pharmaceutical Chemistry
University of Erlangen-Nuernberg
91054 Erlangen
Germany

S. MENZEL*†
R. WAIBEL‡
K. BRUNE†
G. GEISSLINGER†

REFERENCES

1. Caldwell J, Hutt AJ and Fournel-Gigleux S, The metabolic chiral inversion and dispositional enantioselectivity of the 2-arylpropionic acids and their biological consequences. *Biochem Pharmacol* **37**: 105–114, 1988.
2. Nakamura Y, Yamaguchi T, Takahashi S, Hashimoto S, Iwatani K and Nakagawa Y, Optical isomerization mechanism of R(-)-hydratropic acid derivatives. *J Pharmacobiodyn* **4**: s-1, 1981.
3. Knights KM, Drew R and Meffin PJ, Enantiospecific formation of fenoprofen coenzyme A thioester *in vitro*. *Biochem Pharmacol* **37**: 3539–3542, 1988.
4. Tracy TS, Wirthwein DP and Hall SD, Metabolic inversion of (R)-ibuprofen. Formation of ibuprofenyl-coenzyme A. *Drug Metab Dispos* **21**: 114–120, 1993.
5. Shieh WR and Chen CS, Purification and characterization of novel "2-arylpropionyl-CoA epimerases" from rat liver cytosol and mitochondria. *J Biol Chem* **268**: 3487–3493, 1993.
6. Berg P, Acyl adenylates: an enzymatic mechanism of acetate activation. *J Biol Chem* **222**: 991–1013, 1956.
7. Mao LF, Millington DS and Schulz H, Formation of a free acyl adenylate during the activation of 2-propylpentanoic acid. *J Biol Chem* **267**: 3243–3146, 1992.
8. Berg P, The chemical synthesis of amino acyl adenylates. *J Biol Chem* **233**: 608–611, 1958.
9. Menzel-Soglowek S, Geisslinger G and Brune K, Stereoselective high-performance liquid chromatographic determination of ketoprofen, ibuprofen and fenoprofen in plasma using a chiral α_1-acid glycoprotein column. *J Chromatogr* **532**: 295–303, 1990.
10. Porubek DJ, Sanins SM, Stephens JR, Grillo MP, Kaiser DG, Halstead GW, Adams WJ and Baillie TA, Metabolic chiral inversion of flurbiprofen-CoA *in vitro*. *Biochem Pharmacol* **42**: R1–R4, 1991.
11. Tracy TS and Hall SD, Determination of the epimeric composition of ibuprofenyl-CoA. *Anal Biochem* **195**: 24–29, 1991.
12. Knihinicki RD, Day RO and Williams KM, Chiral inversion of 2-arylpropionic acid non-steroidal anti-inflammatory drugs-II. Racemization and hydrolysis of (*R*)- and (*S*)-ibuprofen-CoA thioesters. *Biochem Pharmacol* **42**: 1905–1911, 1991.
13. Tracy TS and Hall SD, Metabolic inversion of (R)-ibuprofen: epimerization and hydrolysis of ibuprofenyl-coenzyme A. *Drug Metab Dispos* **20**: 322–327, 1992.
14. Müller S, Mayer JM, Etter JC and Testa B, Influence of palmitate and benzoate on the unidirectional chiral inversion of ibuprofen in isolated rat hepatocytes. *Biochem Pharmacol* **44**: 1468–1470, 1992.

* Corresponding author: Sabine Menzel, Department of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen, Universitaetsstr. 22, 91054 Erlangen, Germany.