Biochemical Pharmacology, Vol. 52, pp. 1643–1646, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00563-1

SHORT COMMUNICATION

Inhibition of a Medium Chain Acyl-CoA Synthetase Involved in Glycine Conjugation by Carboxylic Acids

Fumiyo Kasuya, Kazuo Igarashi and Miyoshi Fukui*

FACULTY OF PHARMACEUTICAL SCIENCES, KOBE-GAKUIN UNIVERSITY, KOBE, 651-21, JAPAN

ABSTRACT. Molecular characteristics of carboxylic acids were investigated for the ability to inhibit a purified medium chain acyl-CoA synthetase, using hexanoic acid as a substrate. Salicylic acid, 4-methylsalicylic acid, 2-hydroxynaphthoic acid, and 2-hydroxyoctanoic acid, which do not act as substrates for the medium chain acyl-CoA synthetase, were potent as inhibitors. Valproic acid was not an inhibitor. Salicylic acid, 2-hydroxynaphthoic acid, and 2-hydroxyoctanoic acid inhibited the medium chain acyl-CoA synthetase with K_i values of 37, 5.2, and 500 μM, respectively. 4-Methylsalicylic acid was more potent than salicylic acid. The inhibitory carboxylic acids were competitive with respect to hexanoic acid. The distance of the hydroxyl group from the carboxylic acid group of the benzene ring influenced the inhibitory activity. The hydroxyl group on the carbon adjacent to the carboxylic acid group was required for inhibitory activity. In addition, there was a good correlation between the lipophilicity of the carboxylic acids and the K_i values, suggesting that the lipophilicity of the carboxylic acids is a major determinant for inhibition of the medium chain acyl-CoA synthetase. BIOCHEM PHARMACOL 52;10:1643–1646, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. medium chain acyl-CoA synthetase; inhibition; carboxylic acids; glycine conjugation

Many xenobiotic carboxylic acids undergo conjugation with glycine. This reaction is an important route for detoxification of carboxylic acids, such as aromatic, heteroaromatic, arylacetic, and aryloxyacetic acids. The chemical structure of these acids influences glycine conjugation [1–4], which is readily saturated in the high therapeutic dose range [1, 5, 6]. Then, the limited formation of glycine conjugates is compensated for by an increase in glucuronidation, which is also an essential metabolic route for a variety of carboxylic acids. Thus, glycine conjugation and glucuronidation are effective competing pathways in the biotransformation of several carboxylic acids. Not only small planar, but also larger nonplanar molecules are conjugated with glucuronic acid. However, it is unclear what determines the selection between these metabolic routes. We have been investigating the determinants that regulate glycine conjugation.

The pathway of glycine conjugation consists of two sequential reactions. In the first reaction, the carboxylic acid is activated to a CoA thioester in an ATP-dependent reaction catalyzed by acyl-CoA synthetases [7, 8]. The acyl group is then transferred to the amino group of glycine by acyl-CoA:glycine *N*-acyltransferases [9–11]. However, it is unknown whether the specificity of glycine conjugation is exerted at the activation step and/or at the level of glycine transfer. Medium chain acyl-CoA synthetases have not been completely purified from any mammals. We have purified a medium chain acyl-CoA synthetase from bovine liver and observed a very good correlation between the activity of this enzyme for benzoic acids substituted with electron-donating groups and their glycine conjugation [12]. Formation of acyl-CoA thioesters may be the key step in glycine conjugation of xenobiotic carboxylic acids. Therefore, we planned to characterize the medium chain acyl-CoA synthetase with respect to the nature of substrate–enzyme interaction, using inhibitors of the enzyme.

To characterize the interactions of the inhibitors with the medium chain acyl-CoA synthetase, we determined the kinetics of inhibition by a series of carboxylic acids and analyzed the relationship between the structural features of the inhibitors and the inhibitory activity. The purified medium chain acyl-CoA synthetase is specific not only for medium chain fatty acids, such as hexanoic acid, but also for aromatic acids undergoing conjugation with glycine. Since maximal enzymatic activity was observed with hexanoic acid, studies on inhibition of the medium chain acyl-CoA synthetase were carried out with hexanoic acid as the substrate.

MATERIALS AND METHODS

Materials

Fresh bovine liver was obtained from the Nippon Hum Co. Ltd. (Osaka, Japan). Sephadex G-25 and DEAE-Sephacel were purchased from Pharmacia (Piscataway, NJ, U.S.A.),

* Corresponding author: Fumiyo Kasuya, Ph.D., Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, 518, Arise, Ikawadani, Nishi-ku, Kobe, 651-21, Japan. Tel. 81-78-974-1551; FAX. 81-78-974-5689.
Received 16 April 1996; accepted 17 June 1996.

hydroxyapatite from Nacarai Tesque (Kyoto, Japan), and Reactive-Green 19 from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). CoA, NADH and ATP were obtained from the Kohjin Co. Ltd. (Tokyo, Japan); pyruvate kinase, lactate dehydrogenase, myokinase, and phosphoenolpyruvate were from the Oriental Yeast Co. Ltd. (Tokyo, Japan). Other chemicals used were analytical grade and used as received.

Purification of a Medium Chain Acyl-CoA Synthetase

The medium chain acyl-CoA synthetase was purified from bovine liver mitochondria according to a slight modification of a previously described procedure [12].

The enzyme was solubilized from mitochondria with a solution of Triton X-100 and subsequently precipitated by the addition of ammonium sulfate. The ammonium sulfate fractions (0.26 to 0.38 g/mL) were desalted by a Sephadex G-25 column (3.0 × 45 cm). Fractions containing the activity were applied to a DEAE-Sephacel column (3.5 × 15 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0, 2 mM DTT,† 2 mM $MgCl_2$) containing 10% glycerol. The active fractions eluted with 100 mM KCl were subjected to a hydroxyapatite column (2.5 × 20 cm). The active fractions were eluted in a linear gradient of potassium phosphate (10–150 mM, pH 7.4, 2 mM DTT, 2 mM $MgCl_2$, 10% glycerol). Fractions containing the medium chain acyl-CoA synthetase were further chromatographed on a Reactive-Green 19 column (2.5 × 10 cm) equilibrated with 10 mM Tris–HCl buffer (pH 7.8) containing 2 mM DTT, 2 mM $MgCl_2$ and 20% glycerol. The medium chain acyl-CoA synthetase was eluted with 10 mM Tris–HCl buffer (pH 7.8) containing 100 mM KCl, 2 mM DTT, 2 mM $MgCl_2$, and 20% glycerol. The enzyme was judged to be homogeneous by SDS–10% PAGE.

Assay of the Medium Chain Acyl-CoA Synthetase

The medium chain acyl-CoA synthetase activity was determined using the coupled enzyme assay as previously described [12]. Absorbance of the reaction mixture was measured at 340 nm. When absorbance of the inhibitors interfered with the spectrophotometric assay, the hexanoyl-CoA ester formed was quantified by HPLC with a 5 μm Cosmosil C_8 reversed phased column (4.6 mm i.d. × 150 mm) (Nacarai Tesque). Hexanoyl–CoA was eluted with acetonitrile:10 mM phosphate buffer (pH 5.0) (20:80, v/v) at a flow rate of 0.8 mL/min and detected by absorbance at 260 nm. The retention time of hexanoyl-CoA was 5 min and was consistent with that of the authentic standard.

A series of carboxylic acids were screened by determining the activity of the medium chain acyl-CoA synthetase at inhibitor concentrations of 0.25, 1.0, or 1.5 mM (in a final volume of 2.0 mL). The purified medium chain acyl-CoA synthetase (corresponding to 1–5 μg protein) as preincubated with selected inhibitors for 15 min at 37°. The reaction mixture was added and further incubated at 37° for 30 min. For the coupled enzyme assay, the reaction mixture consisted of hexanoic acid as the substrate (0.32 μmol), NADH (0.36 μmol), ATP (5 μmol), CoA (0.6 μmol), $MgCl_2$ (20 μmol), phosphoenolpyruvate (1 μmol), KCl (0.12 μmol), myokinase (1 unit), pyruvate kinase (1 unit), and lactate dehydrogenase (1 unit) in a final volume of 2 mL of 0.2 M Tris–HCl buffer (pH 8.5). For HPLC analysis, the reaction mixture consisted of hexanoic acid (0.32 μmol), CoA (0.6 μmol), ATP (5 μmol), $MgCl_2$ (20 μmol), and KCl (0.12 μmol) in a final volume of 2.0 mL of 0.2 M Tris–HCl buffer (pH 8.5). The reaction was stopped by the addition of 1.0 mL of cold acetonitrile. Then the mixture was transferred into an Ultrafree C3 (Millipore, Milford, MA, U.S.A.) and centrifuged. Aliquots of the filtrate were subjected to HPLC analysis. Quantitation was based on peak heights of samples and the authentic standard.

The kinetics of inhibition were determined by the coupled enzyme assay as indicated above, using various concentrations of hexanoic acid and each inhibitor. The K_i values were obtained by the Dixon plots that were constructed from the data.

Calculation of log P[2]

The log P values were determined according to the method of Hansch et al. [13, 14] or were obtained from the literature [15, 16].

RESULTS AND DISCUSSION

Table 1 shows the effects of various reagents on medium chain acyl-CoA synthetase activity. Salicylic acid, 4-methylsalicylic acid, 2-hydroxynaphthoic acid, and 2-hydroxyoctanoic acid, i.e., compounds with the hydroxyl substitution, were the best inhibitors. When the hydroxyl group was replaced with a methoxy or an amino group, the inhibitory activity was almost lost. The order of the inhibitory activity depended on the position of the hydroxyl substitution on the benzene ring and was ortho > meta > para. 4-Aminosalicylic acid was a less potent inhibitor and 4-methylsalicylic acid was a more potent than salicylic acid. Replacing the benzene ring with a naphthalene ring, as in 2-hydroxynaphthoic acid, resulted in increasing the inhibitory activity. The inhibitory activity was reduced, compared with that of salicylic acid, by replacement of the carboxylic acid group with a methylalcohol group (2-hydroxybenzylalcohol). Valproic acid, which is a branched medium chain fatty acid, caused no inhibition. This is consistent with the report showing that activities of the enzymes involved in glycine conjugation of benzoic acid are not influenced by valproic acid in rat liver mitochondria [17]. On the contrary, the partially purified medium chain acyl-CoA synthetase has been reported to be inhibited by salicylic acid [18]. However, addition of a hydroxyl group to

† *Abbreviations*: DTT, dithiothreitol; and P, octanol-water partition coefficient.

TABLE 1. Effects of various reagents on medium chain acyl-CoA synthetase

Inhibitor	Activity (% of control)
No inhibitor	100
2-Hydroxybenzoic acid*	32 ± 1
3-Hydroxybenzoic acid†	89 ± 5
4-Hydroxybenzoic acid†	95 ± 3
4-Methyl-2-hydroxybenzoic acid*	19 ± 1
2-Hydroxy-4-aminobenzoic acid‡	82 ± 2
2-Methoxybenzoic acid†	98 ± 2
2-Aminobenzoic acid†	92 ± 1
2-Hydroxynaphthoic acid*	11 ± 1
2-Hydroxyoctanoic acid†	62 ± 3
2-Hydroxyhexanoic acid†	91 ± 2
Valproic acid†	99 ± 2
2-Hydroxybenzylalcohol†	72 ± 6

The enzyme was preincubated with various inhibitors for 15 min at 37°, and the incubation mixture was further incubated with 160 μM hexanoic acid as a substrate in 0.2 M Tris–HCl buffer (pH 8.5) as described in Materials and Methods. Enzyme activity was measured spectrophotometrically by the coupled enzyme assay. The specific activity for hexanoic acid in the absence of the inhibitor was 1.98 μmol/min/mg protein and is expressed as 100%.

* Concentration of the inhibitor in a final volume of 2.0 mL was 0.25 mM.
† Concentration of the inhibitor in a final volume of 2.0 mL was 1.0 mM.
‡ Concentration of the inhibitor in a final volume of 2.0 mL was 1.5 mM.

a fatty acid, as in 2-hydroxyoctanoic acid, inhibited the activity. Decreasing the chain length from eight carbons to six carbons, as in 2-hydroxyhexanoic acid, significantly reduced the inhibitory activity. Therefore, the hydroxyl group on the carbon adjacent to the carboxylic acid group is an important determinant for the inhibitory activity. The enzyme was also inhibited 28% by 0.5 mM iodoacetamide, which reacts with thiol groups. These results suggest that there is an interaction (i.e. intermolecular hydrogen bond formation) between the hydroxy group of the inhibitors and one thiol group of the enzyme which is essential for the activity. Since the pKa values of the aromatic acids used are

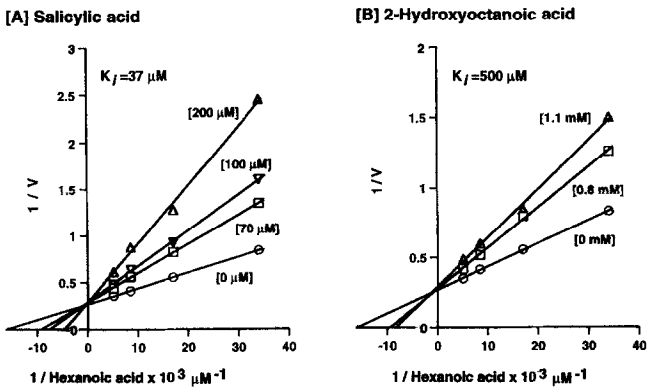

FIG. 1. Inhibition of the medium chain acyl-CoA synthetase activity for hexanoic acid by salicylic acid and 2-hydroxyoctanoic acid. (A) Effect of salicylic acid at concentrations of 0 μM (○), 70 μM (□), 100 μM (▽), and 200 μM (△). (B) Effect of 2-hydroxyoctanoic acid at concentrations of 0 mM (○), 0.8 mM (□), and 1.1 mM (△).

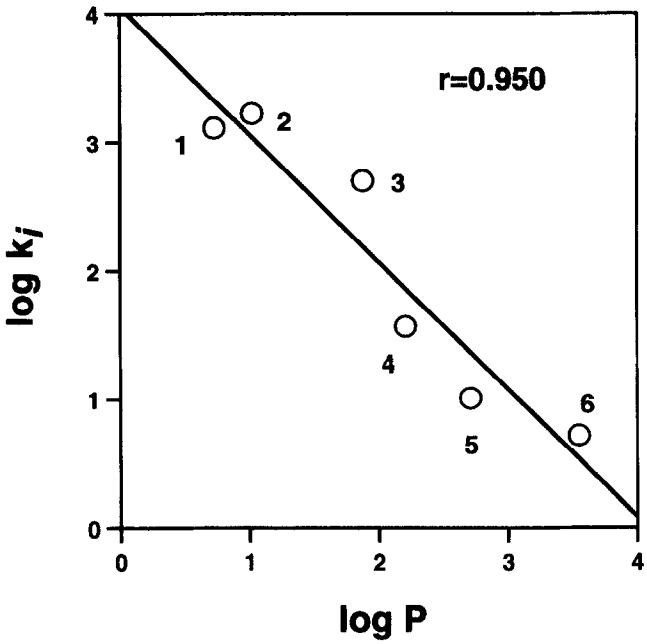

FIG. 2. Correlation between lipophilicity of carboxylic acids and their inhibitory activity for purified medium chain acyl-CoA synthetase. Lipophilicity and inhibitory activity of carboxylic acids are characterized by their octanol-water partition coefficient (P) and K_i values, respectively. Key: (1) 2-hydroxybenzylalcohol; (2) 4-aminosalicylic acid; (3) 2-hydroxyoctanoic acid; (4) salicylic acid; (5) 4-methylsalicylic acid; and (6) 2-hydroxynaphthoic acid.

less than about 5, they are almost completely ionized under our experimental conditions (pH 8.5). Although the pKa values of the medium chain fatty acids are not known, they also appear to be ionized to a similar degree. Therefore, there do not appear to be any effects of pKa on inhibition.

Further, we determined the kinetics of inhibition of the medium chain acyl-CoA synthetase using salicylic acid, 4-methylsalicylic acid, 4-aminosalicylic acid, 2-hydroxynaphthoic acid, 2-hydroxyoctanoic acid, and 2-hydroxybenzylalcohol as inhibitors. Figure 1 shows the Lineweaver–Burk plots for inhibition of the medium chain acyl-CoA synthetase activity for hexanoic acid by salicylic acid and 2-hydroxyoctanoic acid. Formation of hexanoyl-CoA was inhibited competitively by salicylic acid (K_i = 37 μM), 2-hydroxyoctanoic acid (K_i = 500 μM), and 2-hydroxynaphthoic acid (K_i = 5.2 μM). These findings indicate that both the aromatic salicylic acid and the aliphatic 2-hydroxyoctanoic acid can bind to the binding site of hexanoic acid. This finding is also consistent with the fact that both the medium chain fatty acids and aromatic acids serve as substrates for the purified medium chain acyl-CoA synthetase [12]. Of the salicylic acid derivatives, 4-aminosalicylic acid was a weak inhibitor of the enzyme with an apparent K_i value of 1675 μM, whereas 4-methylsalicylic acid was the strongest inhibitor (K_i = 10.2 μM). We observed mixed-type inhibition with 2-hydroxybenzylalcohol (K_i = 1297 μM).

Since the variation in apparent K_i values for the medium

chain acyl-CoA synthetase appeared to follow the hydrophobic character of the inhibitors, we examined the relationship between the lipophilicity of the inhibitors and the K_i values. As seen in Fig. 2, log K_i values decreased with an increase of log P values. 2-Hydroxyoctanoic acid (log P = 1.88) was less potent than salicylic acid (log P = 2.21). In addition, 4-aminosalicylic acid (log P = 1.02) had a weaker inhibitory activity than salicylic acid. In contrast, the relatively lipophilic 4-methylsalicylic acid (log P = 2.71) and 2-hydroxynaphthoic acid (log P = 3.55) were more potent inhibitors than salicylic acid. There was a very good correlation ($r = 0.950$, $P < 0.01$) between lipophilicity of the inhibitors and their K_i values. The result indicates that the lipophilicity of the inhibitors is essential for inhibiting the medium chain acyl-CoA synthetase and that there is hydrophobic interaction between the enzyme and the inhibitors.

References

1. Hutt AJ and Caldwell J, Amino acid conjugation. In: *Conjugation Reactions in Drug Metabolism* (Ed. Mulder GJ), pp. 273–305. Taylor & Francis, London, 1990.
2. Kasuya F, Igarashi K and Fukui M, Glycine conjugation of the substituted benzoic acids *in vitro*: Structure-metabolism relationship study. *J Pharmacobiodyn* **13:** 432–440, 1990.
3. Kasuya F, Igarashi K and Fukui M, Glycine conjugation of the substituted benzoic acids in mice: Structure-metabolism relationship study II. *J Pharmacobiodyn* **14:** 671–677, 1991.
4. Ghauri FY, Blackledge CA, Glen RC, Sweatman BC, Lindon JC, Beddell CR, Wilson ID and Nicholson JK, Quantitative structure–metabolism relationship for substituted benzoic acids in the rat. Computational chemistry, NMR spectroscopy and pattern recognition studies. *Biochem Pharmacol* **44:** 1935–1946, 1992.
5. Patel DK, Ogunbona A, Notarianni LJ and Bennett PN, Depletion of plasma glycine and effect of glycine by mouth on salicylate metabolism during aspirin overdose. *Hum Exp Toxicol* **9:** 389–395, 1990.
6. Gregus Z, Fekete T, Varga F and Klassen CD, Availability of glycine and coenzyme A limits glycine conjugation *in vivo*. *Drug Metab Dispos* **20:** 234–240, 1992.
7. Mahler HR, Wakil SJ and Bock RM, Studies on fatty acid oxidation. 1. Enzymatic activation of fatty acids. *J Biol Chem* **204:** 453–468, 1953.
8. Killenberg PG, Davidson ED and Webster LT, Evidence for a medium chain fatty acid:coenzyme A ligase (adenosine monophosphate) that activates salicylate. *Mol Pharmacol* **7:** 260–268, 1971.
9. Webster LT Jr, Siddiqui UA, Lucas SV, Strong JM and Mieyal JJ, Identification of separate acyl-CoA:glycine and acyl-CoA:L-glutamine *N*-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. *J Biol Chem* **251:** 3352–3358, 1976.
10. Nandi DL, Lucas SV and Webster LT, Benzoyl-CoA:glycine *N*-acyltransferase and phenylacetyl-CoA:glycine *N*-acyltransferase from bovine liver mitochondria: Purification and characterization. *J Biol Chem* **254:** 7230–7237, 1979.
11. Kelley M and Vessey DA, Structural comparison between the mitochondrial aralkyl-CoA and arylacetyl-CoA *N*-acyltransferases. *Biochem J* **288:** 315–317, 1992.
12. Kasuya F, Igarashi K and Fukui M, Participation of a medium chain acyl-CoA synthetase in glycine conjugation of the benzoic acid derivatives with the electron-donating groups. *Biochem Pharmacol* **51:** 805–809, 1996.
13. Hansch C and Clayton JM, Lipophilic character and biological activity of drugs II: The parabolic case. *J Pharm Sci* **62:** 1–21, 1973.
14. Hansch C, Leo A, Unger SH, Kim KH, Nikaitani D and Lien EJ, Aromatic substituent constants for structure–activity correlations. *J Med Chem* **16:** 1207–1216, 1973.
15. Miyake K and Terada H, Determination of partition coefficients of very hydrophobic compounds by high-performance liquid chromatography on glyceryl-coated controlled-pore glass. *J Chromatogr* **240:** 9–20, 1982.
16. Levitan H and Barker JL, Salicylate: A structure–activity study of its effects on membrane permeability. *Science* **176:** 1423–1425, 1972.
17. Gregus Z, Fekete T, Varga F and Klaassen CD, Effect of valproic acid on glycine conjugation of benzoic acid. *J Pharmacol Exp Ther* **267:** 1068–1075, 1993.
18. Londesborough JC and Webster LT Jr, Fatty acyl-CoA synthetase. In: *The Enzymes* (Ed. Boyer PO), 3rd Edn, Vol 10, pp. 469–488. Academic Press, New York, 1974.