Biochemical Pharmacology, Vol. 51, pp. 805–809, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02399-2

Participation of a Medium Chain Acyl-CoA Synthetase in Glycine Conjugation of the Benzoic Acid Derivatives with the Electron-Donating Groups

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ABSTRACT. Glycine conjugation of a series of benzoic acid derivatives was investigated in bovine liver mitochondria. Benzoic acids with chlorine, methyl, methoxy, or ethoxy substituents in the para- or meta-positions of the benzene ring showed a high degree of glycine conjugation. In contrast, the acids with cyano, nitro, amino, or acetylamino groups were conjugated to a small extent with glycine. A medium chain acyl-CoA synthetase that activates carboxylic acids was purified from bovine liver mitochondria. The purified medium chain acyl-CoA synthetase accepted not only medium chain fatty acids but also aromatic and arylacetic acids as substrates. There was a good correlation between the activity of the purified medium chain acyl-CoA synthetase and glycine conjugation of ten benzoic acids with electron-donating substituents. These findings indicate that the purified medium chain acyl-CoA synthetase is a major enzyme for glycine conjugation of benzoic acids with electron-donating groups in bovine liver mitochondria. BIOCHEM PHARMACOL 51;6:805–809, 1996.

KEY WORDS. medium chain acyl-CoA synthetase; substrate specificity; glycine conjugation; benzoic acid derivatives; participation

Glycine and/or glucuronic acid conjugations, which are two parallel competing pathways, are the most important routes of detoxification of many xenobiotic carboxylic acids. At low doses, xenobiotic carboxylic acids tend to undergo amino acid conjugation, but with increasing doses, glucuronic acid conjugation becomes more important [1–3]. Glycine conjugation is readily saturated in the high therapeutic dose range [4]. Thus, compared with glucuronide, conjugation with glycine appears to be of limited capacity. Further, glycine conjugation is restricted to small carboxylic acids, including aromatic, heteroaromatic, arylacetic, and aryloxyacetic acids, whereas glucuronidation is observed toward a very broad range of substrates [1]. There is a need to understand what determines the metabolic fate of xenobiotic carboxylic acids. As a first step, we investigated the determinants that regulate glycine conjugation.

Glycine conjugation proceeds through a two-step reaction [5]. The carboxylic acid is first converted to a high-energy CoA thioester by medium chain acyl-CoA synthetases (EC 6.2.1.2). The activated acid is then transferred from the CoA to the amino group of glycine in a reaction catalyzed by acyl-CoA:glycine *N*-acyltransferases. Depletion of cosubstrates (glycine and CoA) may limit the capacity of glycine conjugation *in vivo* [6]. However, the medium chain acyl-CoA synthetases may also limit glycine conjugation, possibly by regulation of substrate availability for them. Because the formation of the medium chain acyl-CoA thioester may be the key step in the glycine conjugation of xenobiotic carboxylic acids, it is necessary to elucidate the substrate specificity of the medium chain acyl-CoA synthetase and to determine whether the activity of the medium chain acyl-CoA synthetase limits the rate of glycine conjugation. However, the medium chain acyl-CoA synthetases have not been obtained in a highly purified form from any mammals. We have isolated one of the medium chain acyl-CoA synthetases from bovine liver mitochondria.† Then, we investigated the activity of this enzyme for various carboxylic acids, including substituted benzoic acids. In addition, we examined the glycine conjugating activity of bovine liver mitochondria for the substituted benzoic acids and the correlation between the activity of the medium chain acyl-CoA synthetase for the substituted benzoic acids and their glycine conjugation.

MATERIALS AND METHODS

Materials

Fresh bovine liver was obtained from Nippon Hum Co. Ltd. (Osaka, Japan). A Cosmosil C_8 column (150 mm × 4.5 mm i.d.) was purchased from Nacalai Tesque (Kyoto, Japan); Sephadex G-25 and DEAE-Sephacel were obtained from Pharma-

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Received 19 July 1995; accepted 18 October 1995.

† Kasuya F, Igarashi K, Fukui M and Nokihara K, Manuscript submitted for publication.

cia (Piscataway, NJ, U.S.A.), and hydroxylapatite was from Naalai Tesque. A Reactive-Green 19 was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), CoA, NADH and ATP were from Kohjin Co. Ltd. (Tokyo, Japan), and pyruvate kinase, lactate dehydrogenase, myokinase as well as phosphoenolpyruvate were from Oriental Yeast Co. Ltd. (Tokyo, Japan). Other chemicals used were of analytical grade.

Purification of the Medium Chain Acyl-CoA Synthetase†

Mitochondria were prepared from fresh bovine liver (100 g). The mitochondrial pellet obtained was resuspended in 10 mM Tris–HCl buffer (pH 7.8) containing 2 mM DTT,‡ 2 mM $MgCl_2$ and 1% Triton X-100. The suspension was stirred at 4° for 1 hr, and then centrifuged at 17,000 *g* for 30 min. The ammonium sulfate fractions (0.26 to 0.38 g/mL) were collected and desalted by a Sephadex G-25 column (2.5 × 40 cm). The fractions with the medium chain acyl-CoA synthetase activity were chromatographed on a DEAE-Sephacel column (3.5 × 20 cm). The medium chain acyl-CoA synthetase was eluted with 100 mM KCl in 10 mM Tris-HCl buffer (pH 7.8, 2 mM DTT, 2 mM $MgCl_2$). Fractions containing the activity of the medium chain acyl-CoA synthetase from the above column were then applied to a hydroxylapatite column (2.5 × 20 cm). The medium chain acyl-CoA synthetase was eluted in a linear gradient of potassium phosphate (10–150 mM). Fractions containing the medium chain acyl-CoA synthetase were chromatographed further on a Reactive-Green 19 column (2.5 × 10 cm). The active fractions were eluted with 100 mM KCl in 10 mM Tris–HCl buffer (pH 7.8, 2 mM DTT, 2 mM $MgCl_2$, 10% glycerol).

Assay of Glycine Conjugation

Formation of glycine conjugates by bovine liver mitochondria was assayed as described previously [7, 8]. All experiments were performed without the addition of CoA and K^+ since glycine conjugation was sufficiently formed under the experimental conditions without them. This is because the concentrations of CoA and K^+ in the mitochondrial suspension are sufficient for this reaction.

Briefly, incubation mixtures (2.60 mL) consisted of each benzoic acid derivative (0.3 μmol), ATP (15 μmol), glycine (60 μmol), $MgCl_2$ (3 μmol), and mitochondria (corresponding to 3–8 mg protein) in 0.2 M Tris–HCl buffer (pH 8.4). After incubation for 1 hr at 37°C, the reaction was stopped by the addition of 0.35 mL of 7 M HCl. After centrifugation, 0.35 mL of 7 M NaOH was added to the supernatant. Aliquots of the neutralized supernatant were subjected to HPLC analysis. The HPLC analyses of the substituted benzoic acids were carried out as described previously [8]. Before the analysis, 3-amino- and 4-amino-benzoic acids and their glycine conjugates were acetylated by shaking 0.5 mL of the neutralized supernatant with 30 μL of acetic anhydride for 15 min. The formation rates of glycine conjugates were calculated from the disappearance of the substrate.

Assay of the Medium Chain Acyl-CoA Synthetase

The activities of the purified medium chain acyl-CoA synthetase with various carboxylic acids were determined by a coupled enzyme assay, which monitors the formation of AMP.† The reaction mixture consisted of each carboxylic acid (1 μmol), NADH (0.36 μmol), CoA (0.6 μmol), ATP (5 μmol), $MgCl_2$ (20 μmol), phosphoenolpyruvate (1 μmol), KCl (0.12 μmol), myokinase (1 U), pyruvate kinase (1 U), lactate dehydrogenase (1 U) in 1.5 mL of 0.2 M Tris–HCl buffer (pH 8.5). The reaction was initiated by the addition of the medium chain acyl-CoA synthetase (corresponding to 2–6 μg protein). After incubation at 37°C for 30 min, absorbance of the mixture was monitored at 340 nm.

RESULTS AND DISCUSSION

Glycine conjugation, a detoxification pathway for a wide range of exogenous carboxylic acids, occurs in both liver and kidney. Glycine conjugation varies with the animal species and the structure of the acids [1, 7, 8]. Formation of the glycine conjugate is proceeded by the activation of the acid and the transfer of the acyl group. The two stages in the reaction sequence should be investigated individually to determine the regulation of glycine conjugation. Two acyl-CoA:amino acid *N*-acyltransferases have been purified [9–12]. One transferase was found to use benzoyl-CoA, salicyl-CoA, and fatty acyl-CoA esters as substrates. The other acted on phenylacetyl-CoA and indolylacetyl-CoA. Glycine was the acyl acceptor for both enzymes, but glutamine served only for the latter enzyme. Two mammalian medium chain acyl-CoA synthetases that activate aromatic acids, have been purified partially [13, 14]. Purification of benzoyl-CoA ligases from a bacteria, which are different from the medium chain acyl-CoA synthetase from mammals, has been reported [15, 16]. However, medium chain acyl-CoA synthetases have not been isolated in a highly purified form from mammals. Little is known about medium chain acyl-CoA synthetases. Therefore, we have purified a medium chain acyl-CoA synthetase from bovine liver mitochondria to homogeneity and examined the specificity of the enzyme not only for aliphatic carboxylic acids but also for substituted benzoic acids, which are known to undergo glycine conjugation in a variety of species. Thus, we have selected a variety of small carboxylic acids that may be conjugated with glycine. The range of substrates used was limited so that the least number of *N*-acyltransferases would be involved in the transfer step of glycine conjugation.

The relative activities of the medium chain acyl-CoA synthetase for various substrates are shown in Table 1. The substrate specificity of the purified medium chain acyl-CoA synthetase was similar to the enzyme purified partially by Mahler *et al.* [13]. The purified enzyme was specific not only to medium chain fatty acids but also to aromatic carboxylic acids and arylacetic acids. The enzyme activities were observed with the straight medium chain fatty acids. Maximal activity was found with hexanoic acid. Valproic acid (a branched medium chain fatty acid), 2-hydroxyhexanoic acid, and 2-hydroxyoc-

tanoic acid were very poor substrates. High activities were also obtained with benzoic acids substituted with methyl, methoxy, or ethoxy groups in the para- or meta-positions of the benzene ring. The enzyme also exhibited high activities for benzoic acids with large substituents, such as *n*-pentyl, *n*-heptyl, and phenoxy groups. In contrast, benzoic acids with chlorine, cyano, nitro, amino, or acetylamino groups in the para- or meta-positions were poor substrates. Substitution of benzoic acids in the ortho position abolished the enzymatic activity. In the case of arylacetic acids, the enzyme was less active with 1- and 2-naphthylacetic acids as well as with 3-indolylacetic acid.

Table 2 shows glycine conjugation of benzoic acid derivatives formed in bovine liver mitochondria. The chlorine and methyl substitutions in the para- or meta-positions of the benzene ring yielded a marked increase in glycine conjugation. The acids with methoxy or ethoxy groups showed a high degree of glycine conjugation. However, the acids having cyano, nitro, amino, or acetylamino groups were conjugated to a small extent with glycine. The extent of glycine conjugation of the meta-isomers except for chlorine and methyl isomers was greater than that of the para-isomers. In contrast, we reported that the formation rates of the glycine conjugates of meta-isomers and para-isomers were similar in rat liver mitochondria [7]. In mouse liver mitochondria, the degree of glycine conjugation of para-isomers is slightly greater than that of the meta-isomers [8]. Species differences in the extent of glycine conjugation of substituted benzoic acids *in vitro* have been observed [7, 8]. Glycine conjugation of meta-isomers, in particular, varies with species (bovine, mouse, and rat). In spite of the fact that 3-phenoxybenzoic acid is a type of benzoic acid derivative, it was not conjugated with glycine in bovine liver mitochondria. However, the purified enzyme displayed high activity for 3-phenoxybenzoic acid (Table 1). 3-Phenoxybenzoic acid has been reported to undergo conjugation with taurine in the mouse, with glucuronic acid in the rat [17], and with glutamic acid in the cow [1].

TABLE 1. Substrate specificity of the medium chain acyl-CoA synthetase

Substrate	Relative activity (%)
Propionic acid	8
Hexanoic acid	100
Octanoic acid	83
Dodecanoic acid	39
2-Hydroxyhexanoic acid	6
2-Hydroxyoctanoic acid	3
Valproic acid	4
Benzoic acid	19
4-Chloro-	17
4-Cyano-	3
4-Nitro-	1
4-Methyl-	59
4-Amyl-	39
4-Heptyl-	66
4-Methoxy-	47
4-Ethoxy-	22
4-Amino-	3
4-Acetylamino-	2
3-Chloro-	12
3-Cyano-	4
3-Nitro-	1
3-Methyl-	23
3-Methoxy-	48
3-Phenoxy-	44
3-Amino-	5
3-Acetylamino-	2
2-Hydroxy-	< 1
2-Methoxy-	0
2-Chloro-	0
2-Iodo-	0
1-Naphthylacetic acid	18
2-Naphthylacetic acid	21
3-Indolylacetic acid	15

The specific activity for hexanoic acid was 477 nmol/min/mg protein. Activities are expressed as percent of the specific activity of hexanoic acid. Values represent the averages of three experiments.

TABLE 2. Glycine conjugation of the substituted benzoic acids in bovine liver mitochondria

Substrate	Relative activity (%)
Benzoic acid	100
4-Chloro-	162
4-Cyano-	41
4-Nitro-	29
4-Methyl-	174
4-Methoxy-	96
4-Ethoxy-	82
4-Amino-	17
4-Acetylamino-	7
3-Chloro-	141
3-Cyano-	47
3-Nitro-	74
3-Methyl-	110
3-Methoxy-	129
3-Amino-	38
3-Acetylamino-	0

The specific activity for benzoic acid was 24.8 nmol/mg protein. Activities are expressed as percent of the specific activity of benzoic acid. Values represent the averages of three experiments.

Arylacetic acids tend to be conjugated with glutamine or taurine rather than glycine. Therefore, we did not examine glycine conjugation of arylacetic acids. 1-Naphthylacetic acid is conjugated with glycine *in vivo* in the rat and mouse, and with glutamine in humans [1, 18]. The amino acids utilized in an *in vivo* conjugation of 2-naphthylacetic acid, are mainly taurine in the mouse, glycine in the rat, and glutamine in humans [3, 18, 19]. 3-Indolylacetic acid is conjugated with glycine *in vivo* in the rat, and with glutamine in Old World and New World monkeys and in humans [20]. However, acyl-CoAs are essential intermediates for the conjugation of carboxylic acids with any amino acids. Because arylacetic acids were relatively poor substrates for the medium chain acyl-CoA synthetase purified in the present work, it is assumed that other medium chain acyl-CoA synthetases activate arylacetic acids.

Glycine conjugates of several branched medium chain fatty acids have been observed as abnormal urinary metabolites in

subjects with medium chain acyl-CoA dehydrogenase deficiency [21]. However, the fatty acyl-CoAs that are formed by the medium chain acyl-CoA synthetase are usually degraded via β-oxidation.

The extent to which the purified medium chain acyl-CoA synthetase is involved in the initial step of glycine conjugation was assessed by investigating the relationship between the activity of the purified medium chain acyl-CoA synthetase and glycine conjugation of sixteen substituted benzoic acids in bovine liver mitochondria (Fig. 1). The linear regression analysis demonstrated a correlation ($r = 0.725$) between glycine conjugation and the activity of the medium chain acyl-CoA synthetase for sixteen benzoic acid derivatives. The acids having the electron-withdrawing substituents, such as chlorine, cyano or nitro groups, showed a higher degree of glycine conjugation, compared with the low activities of the medium chain acyl-CoA synthetase for them. Therefore, the acids with the electron-withdrawing groups are responsible for the low correlation coefficient. On the other hand, there was a good correlation ($r = 0.914$, $P < 0.01$) between glycine conjugation and the activity of the medium chain acyl-CoA synthetase for ten acids having the electron-donating groups including benzoic acid (Fig. 2). These findings indicate that the purified enzyme plays a major role in glycine conjugation of the acids with the electron-donating groups in bovine liver mitochondria. In the case of the acids with the electron-withdrawing groups, other isozymes may catalyze the initial reaction of the formation of glycine conjugates in bovine liver mitochondria. However, in spite of low activities of the medium chain acyl-CoA synthetase for the acids with the electron-withdrawing groups, it is possible that the same medium chain acyl-CoA synthetase is predominantly responsible for their glycine conjugation. This would be supported by the existence of the acyl-CoA:glycine *N*-acyltransferase, which shows very high activities not toward the CoA esters of the acids having electron-donating groups but toward those having electron-withdrawing groups. To date, only one acyl-CoA:glycine *N*-acyltransferase in liver mitochondria, which conjugates substituted benzoyl-CoAs and medium chain acyl-CoAs with glycine, has been reported. The extent to which the various substituents affect the activity of *N*-acyltransferase is unclear. The number of enzymes catalyzing glycine conjugation has not yet been elucidated. Differences between the substrate specificity of the purified enzyme and overall *in vivo* amino acid conjugation reported previously are apparent. The result suggests that there are some enzymes overlapping the substrate specificities. To elucidate further the roles of medium chain acyl-CoA synthetases in the regulation of glycine conjugation, we are currently investigating the purification of another isozyme activating salicylic acid.

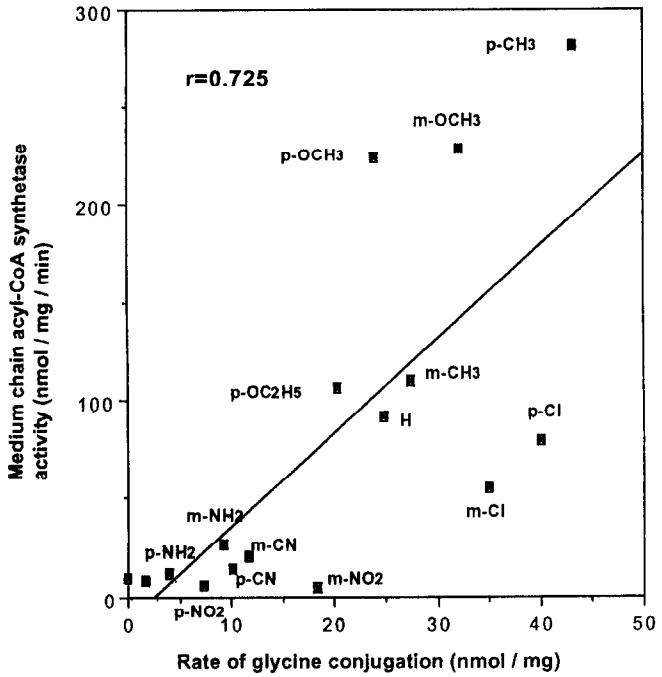

FIG. 1. Correlation between the activity of the medium chain acyl-CoA synthetase and glycine conjugation of the substituted benzoic acids. The activities of the medium chain acyl-CoA synthetase for the substituted benzoic acids and their glycine conjugation were determined as described in Materials and Methods. Results are averages of three experiments.

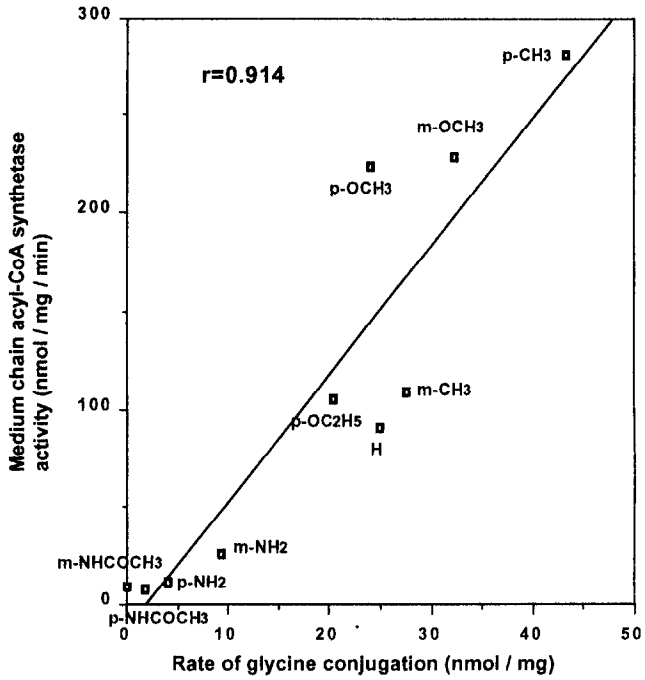

FIG. 2. Correlation between the activity of the medium chain acyl-CoA synthetase and glycine conjugation of the substituted benzoic acids with the electron-donating groups. The activities of the medium chain acyl-CoA synthetase for the substituted benzoic acids and their glycine conjugation were determined as described in Materials and Methods. Results are averages of three experiments. The correlation was statistically significant at $P < 0.01$.

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