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Biochemical Pharmacology

Biochemical Pharmacology 62 (2001) 363–367

Short communication

Inhibitory effect of quinolone antimicrobial and nonsteroidal anti-inflammatory drugs on a medium chain acyl-CoA synthetase

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Received 15 August 2000; accepted 28 November 2000

Abstract

The inhibitory effects of quinolone antimicrobial agents and nonsteroidal anti-inflammatory drugs on purified mouse liver mitochondrial medium chain acyl-CoA synthetase catalyzing the first reaction of glycine conjugation were examined, using hexanoic acid as a substrate. Enoxacin, ofloxacin, nalidixic acid, diflunisal, salicylic acid, 2-hydroxynaphthoic acid, and 2-hydroxydodecanoic acid, which do not act as substrates, were potent inhibitors. Diflunisal, nalidixic acid, salicylic acid, 2-hydroxynaphthoic acid, and 2-hydroxydodecanoic acid inhibited competitively this medium chain acyl-CoA synthetase with K_i values of 0.6, 12.4, 19.6, 13.4, and 15.0 μM, respectively. Enoxacin and ofloxacin inhibited this medium chain acyl-CoA synthetase in a mixed-type manner with K_i values of 23.7 and 38.2 μM, respectively. Felbinac, which is a substrate, inhibited the activity of this medium chain acyl-CoA synthetase for hexanoic acid (IC_{50} = 25 μM). The concomitant presence of enoxacin and felbinac strongly inhibited this medium chain acyl-CoA synthetase. These findings indicate that medium chain acyl-CoA synthetases may be influenced by quinolone antimicrobial and nonsteroidal anti-inflammatory drugs.

Keywords: Medium chain acyl-CoA synthetase; Inhibition; Quinolone antimicrobial drug; Nonsteroidal anti-inflammatory drug

1. Introduction

Glycine conjugation is one of the most important routes for detoxification, not only of many xenobiotic carboxylic acids but also of endogenous acids. Glycine conjugation proceeds through a two-step reaction sequence [1]. The carboxylic acid is initially converted to a high-energy CoA thioester by medium chain acyl-CoA synthetases and then is transferred from CoA to the amino group of glycine in a reaction that is catalyzed by acyl-CoA:glycine *N*-acyltransferases. The latter are known to occur in two distinct forms that have been purified to homogeneity and characterized extensively [2–4]. However, little information is available about the medium chain acyl-CoA synthetases catalyzing the initial reaction of glycine conjugation. At least three medium chain acyl-CoA synthetases have been partially purified from liver mitochondria [5–8]. We recently purified one medium chain acyl-CoA synthetase from bovine liver [9,10] and mouse kidney mitochondria [11], which did not utilize salicylic acid and diflunisal as substrates. To demonstrate the possible mechanisms of side-effects induced by the drugs, it is necessary to elucidate the effects of a series of drugs on each metabolizing enzyme. We have been investigating the interaction between the drugs and this purified mouse liver mitochondrial medium chain acyl-CoA synthetase.

There are many reports about side-effects induced by NSAIDs. For example, salicylate and aspirin have been identified as being associated with the development of Reye's syndrome [12]. As a possible role in the development of Reye's syndrome, salicylic acid was reported to decrease the mitochondrial activation and the β-oxidation of long chain fatty acids (*in vivo*) [13], and to inhibit the β-oxidation of medium chain fatty acids (*in vitro*) [14]. Medium chain acyl-CoA dehydrogenase deficiency, a commonly inherited metabolic disorder affecting β-oxidation of fatty acids, causes a Reye's syndrome-like disease [15,16]. Therefore, Reye's syndrome may be connected in some way with a disorder in fatty acid metabolism. We have shown

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Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; NQs, quinolone antimicrobial drugs; DTT, dithiothreitol; and GABA, γ-aminobutyric acid.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00667-0

that salicylic acid and diflunisal are potent inhibitors of a medium chain acyl-CoA synthetase, which accepted medium chain fatty acids as substrates with hexanoic acid showing maximal activity [9,11,17]. The inhibition of medium chain acyl-CoA synthetase might also be a possible mechanism for the development of Reye's syndrome. On the other hand, we have reported that the hydroxyl group at the β-position of carboxylic acids, as found in salicylic acid and diflunisal, is an important structural feature for inhibiting the medium chain acyl-CoA synthetase from bovine liver mitochondria [17]. The explanation is that orientation of the β-hydroxyl group of the inhibitors might result in the formation of a hydrogen bond with one thiol group, which has been proven to be important in the catalytic activity of this medium chain acyl-CoA synthetase [9]. Salicylic acid derivatives have their hydroxyl group at the β-position relative to the carboxyl group, whereas NQs possess a ketone (oxo) group at this position. Therefore, NQs are accessible to the active site of the enzyme in a manner similar to NSAIDs. We plan to elucidate the effects of NQs on medium chain acyl-CoA synthetase. NSAIDs and NQs are known to cause adverse reactions (e.g. convulsions induced by the interaction of NQs and NSAIDs) [18–20]. It is important to explore the possible mechanisms of the side-effects induced by NSAIDs and NQs.

We selected medium chain acyl-CoA synthetase purified from mice because the mouse is frequently used as the experimental model in these types of studies. No information is available about the medium chain acyl-CoA synthetase from mouse liver mitochondria. We examined the effects of NSAIDs and NQs on the activity of mouse liver mitochondrial medium chain acyl-CoA synthetase and determined the kinetics of inhibition.

2. Materials and methods

2.1. Materials

Sephadex G-25 and DEAE-Sepharose were purchased from Pharmacia, and Reactive-Green 19 and hexanoyl-CoA from the Sigma Chemical Co. Hydroxyapatite was obtained from Nacalai Tesque. CoA, NADH, and ATP were purchased from the Kohjin Co. Ltd., and pyruvate kinase, lactate dehydrogenase, myokinase, and phosphoenolpyruvate from the Oriental Yeast Co. Ltd. The other chemicals used were of analytical grade and were used as received.

2.2. Purification of a medium chain acyl-CoA synthetase

A medium chain acyl-CoA synthetase was purified from the liver (96 g) of ddY strain mice (6 weeks, 25–28 g) as described previously [17]. Briefly, 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 passage through a Sephadex G-25 column (2.0 $\times$ 45 cm). Fractions containing enzyme activity were applied to a DEAE-Sepharose column (3.5 $\times$ 5 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0), containing 2 mM DTT, 2 mM $MgCl_2$, and 10% glycerol. The active fractions eluted with 100 mM KCl and then were passed through a hydroxyapatite column (2.0 $\times$ 13 cm). The active fractions were eluted with 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 chromatographed further on a Reactive-Green 19 column (1.5 $\times$ 3 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.

2.3. Assay of the mouse liver mitochondrial medium chain acyl-CoA synthetase

The activity of this medium chain acyl-CoA synthetase was determined using the coupled enzyme assay as described previously [17]. Absorbance of the reaction mixture was measured at 340 nm.

We selected a series of drugs having hydroxyl or ketone (oxo) groups at the β-position of carboxylic acids as the structural features of inhibitors and did a preliminary examination of the inhibitory effect of a 0.25 mM concentration of selected carboxylic acids. The reaction mixture was incubated with each inhibitor 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 (29 μmol), myokinase (2 U), pyruvate kinase (2 U), lactate dehydrogenase (2 U), and the medium chain acyl-CoA synthetase (corresponding to 1–5 μg protein) in a final volume of 2 mL of 0.2 M Tris–HCl buffer (pH 8.5).

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 from Dixon plots.

2.4. HPLC analysis of hexanoyl-CoA

To examine the inhibitory ability of felbinac, the formation of hexanoyl-CoA was analyzed by HPLC equipped with a 5-μm Cosmosil C_{18}-MS column (4.6 mm i.d. $\times$ 150 mm) (Nacalai Tesque). Hexanoyl-CoA was eluted with acetonitrile:10 mM ammonium acetate buffer (pH 5.3) (14:86, 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 10 min, and was consistent with that of the authentic standard. The reaction mixture consisted of hexanoic acid (0.1

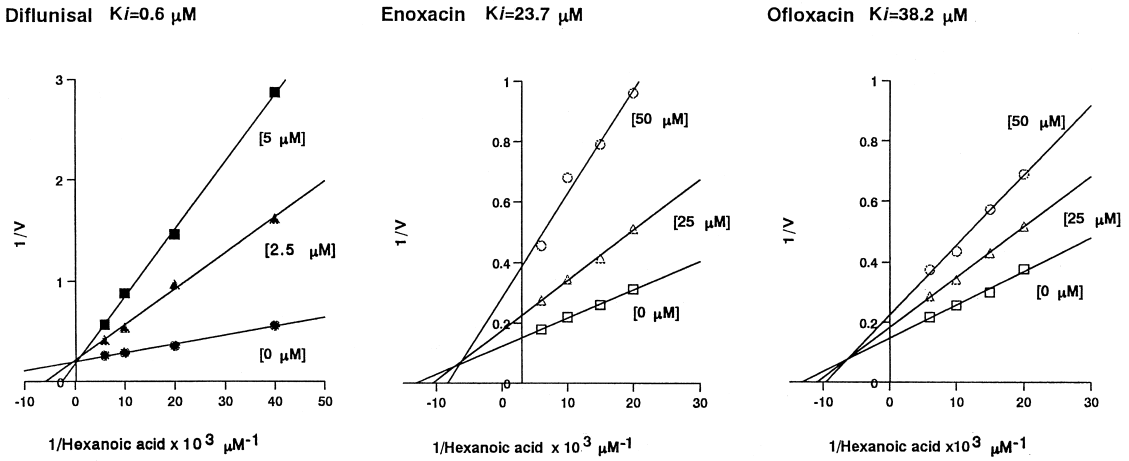

Fig. 1. Inhibition of medium chain acyl-CoA synthetase activity for hexanoic acid by diflunisal, enoxacin, and ofloxacin. (Left panel): Effect of diflunisal at 0 μM (●), 2.5 μM (▲), and 5 μM (■). (Center panel): Effect of enoxacin at 0 μM (□), 25 μM (△), and 50 μM (○). (Right panel): Effect of ofloxacin at 0 μM (□), 25 μM (△), and 50 μM (○). The K_i values are the means from three experiments.

μmol), CoA (0.6 μmol), ATP (5 μmol), $MgCl_2$ (20 μmol), KCl (29 μmol), and each inhibitor (10–100 μM), and was incubated at 37° for 30 min. After incubation was stopped by the addition of 0.1 mL of cold acetonitrile and 0.3 mL of 0.5 M sodium citrate–HCl buffer (pH 2.0), the mixture was centrifuged at 15,000 *g* for 15 min at 4°, and an aliquot of the supernatant was subjected to HPLC analysis. Quantitation was based on the peak area of the samples and the authentic standard.

2.5. Protein analysis

Protein was determined in duplicate at 595 nm with a protein assay kit (Bio-Rad).

3. Results and discussion

The medium chain acyl-CoA synthetase from mouse liver mitochondria was purified using the same method as used previously for the bovine liver mitochondria [17]. The active fractions from a Reactive-Green 19 column were analyzed by SDS–PAGE. The protein band was visualized by Coomassie brilliant blue and corresponded to one major band of 65 kDa. Enzymatic activity was found for medium chain fatty acids, with hexanoic acid showing the maximal activity. In addition, the enzyme accepted aromatic and arylacetic acids as substrates. Hexanoic acid exhibited four times higher activity than benzoic acid. High activities were obtained with benzoic acid derivatives having methyl or methoxy groups in the para- and meta-positions of the benzene ring. The enzyme was less active with acids having nitro or amino groups in the para- or meta-positions. Substitution of benzoic acids in the ortho-position, as in salicylic acid, abolished the activity. In the case of arylacetic acids, 1- and 2-naphthylacetic acids and felbinac exhibited almost the same activity as benzoic acid. Ketoprofen was a very poor substrate. The substrate specificity of mouse liver mitochondrial medium chain acyl-CoA synthetase was very similar to the enzyme activity found previously in mouse kidney [11].

To further elucidate whether mouse liver mitochondrial medium chain acyl-CoA synthetase is affected by NSAIDs (diflunisal, salicylic acid, and felbinac) and NQs (nalidixic acid, enoxacin, and ofloxacin), we determined the kinetics of the inhibition of medium chain acyl-CoA synthetase. The inhibitory effects of 2-hydroxynaphthoic and 2-hydroxydodecanoic acids were also compared with results obtained previously using the enzyme isolated from bovine liver. Diflunisal, enoxacin, and ofloxacin, which did not serve as substrates, inhibited the activity of this mouse liver mitochondrial medium chain acyl-CoA synthetase for hexanoic acid, as shown in the Lineweaver–Burk plots presented in Fig. 1. Formation of hexanoyl-CoA was inhibited competitively by diflunisal (K_i = 0.6 μM), 2-hydroxynaphthoic acid (K_i = 13.4 μM), 2-hydroxydodecanoic acid (K_i = 15.0 μM), and salicylic acid (K_i = 19.6 μM). In the case of carboxylic acids having a ketone (oxo) group at the β-position, nalidixic acid (K_i = 12.4 μM) competitively inhibited this medium chain acyl-CoA synthetase, whereas we observed mixed-type inhibition by enoxacin (K_i = 23.7 μM) and ofloxacin (K_i = 38.2 μM). The order of inhibitory activity for the enzyme from bovine liver mitochondria was diflunisal (K_i = 0. 8 μM) > 2-hydroxydodecanoic acid (K_i = 4.4 μM) > 2-hydroxynaphthoic acid (K_i = 5.2 μM) > salicylic acid (K_i = 37.0 μM) [17]. Similar K_i values were obtained for the medium chain acyl-CoA synthetase from mouse liver mitochondria. The enzymes from mouse liver and bovine liver had similar sensitivities to these inhibitors. NSAIDs and NQs having hydroxyl or ketone (oxo) groups at the β-position inhibited the activity of the medium chain acyl-CoA synthetase. These results suggest that there is an

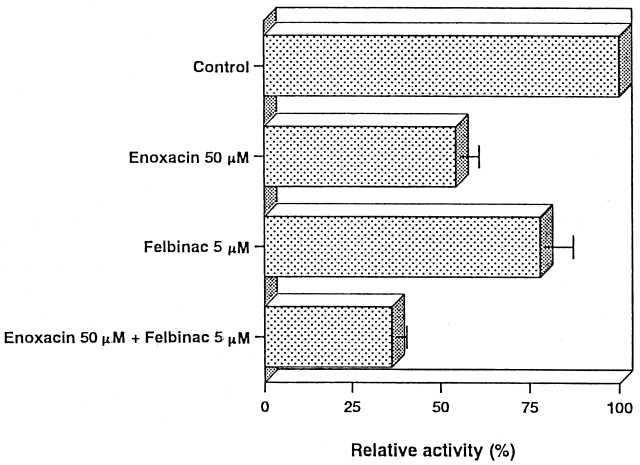

Fig. 2. Effects of enoxacin and felbinac on medium chain acyl-CoA synthetase activity for hexanoic acid. The specific activity for hexanoic acid in the absence of the inhibitors was 910 nmol/mg/min and was expressed as 100%. Values are means ± SD from 4 different mitochondrial samples.

interaction (i.e. intermolecular hydrogen bond formation) between the ketone group of the inhibitors and one thiol group of this enzyme that is essential for enzyme inhibition by the same mechanism as proposed for the β-hydroxyl group of NSAIDs.

There are reports about side-effects induced by the interaction of NQs and NSAIDs. The incidence of convulsions induced by NQs increased with concomitant administration of NSAIDs [18–20]. A widely accepted mechanism for the onset of convulsions involves the blockage of GABAergic neurotransmission in the CNS. Concomitant administration of enoxacin and felbinac was the most likely combination to cause convulsions. The inhibitory effect of enoxacin (IC_{50} = 58 μM) on the GABA response was stronger than that of ofloxacin (IC_{50} = 280 μM) [20]. The order of the inhibitory activity for the medium chain acyl-CoA synthetase was the same as that for the GABA response.

We examined the extent to which this medium chain acyl-CoA synthetase is affected by enoxacin and felbinac. Felbinac, which is a substrate for this medium chain acyl-CoA synthetase, exhibited almost the same activity as benzoic acid. Therefore, the activity of this medium chain acyl-CoA synthetase for hexanoic acid was determined using HPLC. Formation of hexanoyl-CoA was inhibited 45.7% by 50 μM enoxacin and 22.1% by 5 μM felbinac (IC_{50} = 25 μM) (Fig. 2). The combination of 50 μM enoxacin and 5 μM felbinac inhibited this mouse liver mitochondrial medium chain acyl-CoA synthetase activity by 63.9%. We did not demonstrate that the inhibition of this medium chain acyl-CoA synthetase by NQs and NSAIDs causes convulsions. However, the effects of NQs and NSAIDs on this medium chain acyl-CoA synthetase were very similar to those on the GABA response. The inhibition of the medium chain acyl-CoA synthetase by NSAIDs and NQs might be one of other possible mechanisms for the induction of convulsions. These findings were obtained by studying the inhibition of only one medium chain acyl-CoA synthetase. No data are available about the inhibition of the salicylate-activating enzyme. Whether the medium chain acyl-CoA synthetases are located in the brain is unclear. It will also be necessary to investigate whether the medium chain acyl-CoA synthetases are inhibited *in vivo* by NQs and NSAIDs.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research.

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