Effect of carboxylic acid xenobiotics and their metabolites on the activity of carnitine acyltransferases

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Salicylyl-CoA and benzoyl-CoA were good inhibitors of carnitine acetyltransferase (CAT), competing with acetyl-CoA with K_i values of 7.5 and 22 μM respectively in the forward direction and with CoA in the reverse reaction with similar K_i values. They were also competitive inhibitors of carnitine octanoyltransferase (K_i = 261 and 295 μM respectively), but were only weakly inhibitory to carnitine palmitoyltransferase. Inhibition of energy production by salicylate may result from the inhibition of CAT by salicylyl-CoA.

INTRODUCTION

A number of carboxylic acid-containing xenobiotics are metabolized inside mitochondria. One of these, salicylic acid, has been shown to interfere with the ability of mitochondria to oxidize fatty acids [1]. This may be the explanation for the observation that salicylate plays a role in the etiology of Reye's syndrome [2–5], a disease characterized by altered fatty acid metabolism [6–9]. In searching for a mechanism for the inhibition of mitochondrial fatty acid oxidation, it is noteworthy that the metabolism of salicylic acid, and certain other carboxylic acids, has a point of overlap with the metabolism of fatty acids. This overlap is the result of their common formation of acyl-CoA compounds, and represents a potential site for carboxylic acids to interfere with fatty acid metabolism. Carboxylic acid xenobiotics which are to be conjugated with amino acids are first activated to the CoA thioester by ATP-dependent ligases in the mitochondrial matrix [10,11]. In addition to tying up the intramitochondrial pool of CoA, these acyl-CoA compounds might compete with either fatty acyl-CoA or CoA for binding to the carnitine acyltransferases and thereby interfere with the normal carnitine-dependent flux of activated fatty acyl groups into the mitochondrial acyl-CoA pool for β-oxidation.

The carnitine acyltransferases mediate the transfer of activated fatty acids into the mitochondria for oxidation and play a critical role in buffering the acylation state of the small CoA pools present in mitochondria and in the cytoplasm (for reviews see [12,13]). Carnitine palmitoyltransferase is found on the mitochondrial outer membrane (CPT-I) and on the inner membrane (CPT-II) of mitochondria. Carnitine octanoyltransferases (COT) with specificity for medium-to-long-chain fatty acids are found in peroxisomes and in microsomes. Carnitine acetyltransferase (CAT), with specificity for short-chain acyl groups, is found in all three locations. In mitochondria, CAT functions to buffer the acetyl-CoA/CoA ratio [12,13]. Inhibition of these transferases results in pathology due to the metabolic consequences of the increased acylation of the limited pool of CoA and to decreased oxidation of fatty acids.

In this paper we have investigated the effect of the CoA and glycine derivatives of salicylic acid and benzoic acid on the activity of three purified carnitine transferases, CAT, COT and CPT-II.

MATERIALS AND METHODS

Benzoic acid, benzoyl-CoA, L-carnitine, salicylic acid, hippuric acid, 2-hydroxyhippuric acid (salicyluric acid), naphthylacetic acid, phenylacetic acid and CAT were obtained from Sigma, St. Louis, MO, U.S.A. Acetylsalicylyl chloride was obtained from Aldrich, Milwaukee, WI, U.S.A. The synthesis of salicylyl-CoA from acetylsalicylyl chloride is described elsewhere [14]. Naphthylacetyl-CoA was synthesized from naphthylacetic acid via the acid chloride as described previously [14].

COT was purified from bovine liver by the method of Ramsay et al. [15]. CPT-II was purified from bovine liver mitochondria by the method of Clarke & Bieber [16].

All three carnitine transferases were assayed in the forward and reverse directions by monitoring the change in thioester absorbance of the fatty acyl-CoA at 232 nm. Assays were conducted at 30 °C. The standard assay for CAT contained: 50 μM-acetyl-CoA, 500 μM-L-carnitine, 65 mM-Hepes, pH 8.0, and 0.06 unit of CAT. The reverse reaction contained: 400 μM-acetylcarnitine, 100 μM-CoA, 65 mM-Hepes, pH 8.0, and 0.06 unit of CAT. The standard assay for COT contained: 10 μM-decanoyl-CoA, 100 μM-L-carnitine, 100 mM-potassium phosphate, pH 7.4, and COT. The reverse reaction contained: 200 μM-decanoyl-L-carnitine, 100 μM-CoA, 100 mM-potassium phosphate, pH 7.4, and COT. The standard assay for CPT-II contained 10 μM-decanoyl-CoA, 200 μM-L-carnitine, 100 mM-potassium phosphate, pH 7.4, and CPT-II. The reverse reactions contained 100 μM-CoA, 350 μM-decanoyl-L-carnitine, 100 mM-potassium phosphate, pH 7.4, and CPT-II. The inhibition kinetics were analysed by the method of Dixon [17].

RESULTS

CAT was tested first for inhibition by a range of carboxylic acid xenobiotics. Benzoic acid, salicylic acid, naphthylacetic acid and phenylacetic acid were without effect on enzyme activity at concentrations up to 0.5 mM. None of the CoA esters of these compounds was found to be a substrate for CAT. The glycine conjugates of benzoic acid and salicylic acid were also tested and were not inhibitory at concentrations up to 0.5 mM. However, both benzoyl-CoA and salicylyl-CoA were potent inhibitors. The data in Fig. 1 reveal that both were competitive inhibitors versus acetyl-CoA. The apparent K_i values determined from Dixon
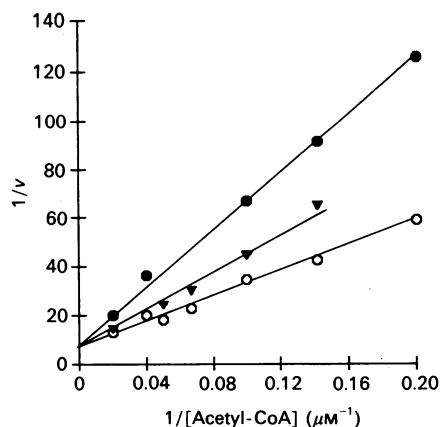

Abbreviations used: CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase.

Fig. 1. Inhibition of CAT by benzoyl-CoA and salicylyl-CoA

CAT was assayed as described in the Materials and methods section with the concentration of carnitine fixed at 500 μM: $\bigcirc$, rates determined in the absence of inhibitor; $\bullet$, rates determined in the presence of 4 μM-salicylyl-CoA; $\blacktriangle$, rates determined in the presence of 20 μM-benzoyl-CoA. The rate of reaction (v) is expressed in absorbance units/min per μl of enzyme.

plots were 22 ± 1 μM for benzoyl-CoA and 7.5 ± 0.5 μM for salicylyl-CoA (Table 1). The apparent K_m for acetyl-CoA at 500 μM-L-carnitine was 31 ± 2 μM. For comparison, we determined the K_i for end-product inhibition by CoA and obtained the value 22 ± 1 μM (Table 1). When the reaction was run in the reverse direction, both benzoyl-CoA and salicylyl-CoA were competitive inhibitors versus CoA. The apparent K_m for CoA was 33 μM. The K_i values were 17 ± 3 μM for benzoyl-CoA and 9.5 ± 0.5 μM for salicylyl-CoA, very similar to the values obtained for inhibition of the forward reaction (Table 1).

The effects of benzoic acid, hippuric acid, salicylic acid and salicyluric acid on peroxisomal COT were tested. None of them was found to be inhibitory at concentrations up to 0.5 mM. Benzoyl-CoA and salicylyl-CoA were weak competitive inhibitors of decanoyl-CoA binding. The K_i values were 295 ± 15 μM for benzoyl-CoA and 261 ± 2 μM for salicylyl-CoA (Table 1), much higher than the K_i for end-product inhibition by CoA ($K_i = 44$ μM). Acetyl-CoA was less inhibitory than either salicylyl-CoA or benzoyl-CoA. The K_m for decanoyl-CoA was 1.3 ± 0.1 μM. In the reverse direction the K_m for CoA was 21 μM [18], but no inhibition by benzoyl-CoA or salicylyl-CoA was detected with 250 μM of either ester.

CPT-II was also examined with these compounds. Again, benzoic acid, hippuric acid, salicylic acid, salicyluric acid, naphthylacetic acid and phenylacetic acid were not inhibitory at 0.5 mM. Benzoyl-CoA was a such a weak inhibitor that a kinetic

analysis could not be done. At 0.25 mM (the highest concentration that could be added to the cuvette, owing to its high A_{232}), benzoyl-CoA inhibited by only 14%. Salicylyl-CoA was also a weak inhibitor, giving an inhibition of 22% at 0.25 mM. The K_m for decanoyl-CoA was 2.5 ± 0.2 μM. In the reverse direction, non-linear kinetics were observed, but again, both benzoyl-CoA and salicylyl-CoA were very weak inhibitors.

DISCUSSION

The data indicate that CAT, the carnitine acyltransferase which uses short-chain acyl-CoA substrates, has the highest affinity for both salicylyl-CoA and benzoyl-CoA. Benzoyl-CoA has about the same K_i as CoA, suggesting that the benzoate group has little effect on binding. Salicylyl-CoA actually has a lower K_i for the enzyme than CoA or benzoyl-CoA. It is not clear why the addition of a hydroxyl group should improve the interaction with the enzyme. COT and CPT-II are specific for medium- and long-chain fatty acyl-CoA derivatives [15]. Although acetyl-CoA is an extremely poor substrate of COT [15], reflecting the specificity of COT to medium- to long-chain acyl-CoA analogues, it did not inhibit COT under the assay conditions used here. It is thus not surprising that the benzoyl and salicylyl moieties are poorly recognized as acyl groups by COT and CPT-II.

Since acyl-CoA compounds such as salicylyl-CoA are formed in the mitochondrial matrix [18], their inhibitory effects will be exerted on CAT and on CPT-II. Our data show that there is no direct inhibition of the CPT-II activity which provides long-chain acyl groups for β-oxidation. However, inhibition of CAT means that the buffering of the acylation state of the small CoA pool cannot take place. Transfer of acetyl and other short-chain acids to the larger pool of carnitine in the matrix (0.2 mM [19]) and, hence, via the carnitine translocase [20] out into the cytoplasm is important, both to maintain a pool of activated acetyl groups for synthetic or oxidative use and to prevent all the matrix CoA from becoming esterified. As acyl-CoA builds up, less CoA is available for β-oxidation cycles or for the formation of succinyl-CoA in the tricarboxylic acid cycle. The fall in the CoA/acetyl-CoA ratio could also inhibit enzymes such as pyruvate dehydrogenase [21]. In Reye's syndrome patients, free CoA levels are decreased to less than 10% of the total pool [22]. Under these conditions, energy production is decreased, and clinical symptoms, such as high circulating concentrations of non-esterified fatty acids, low ketone-body levels and hyper-ammonaemia, can appear.

Table 1. Kinetic constants for the inhibition of the carnitine acyltransferases by xenobiotic CoA derivatives

No I, no inhibition; N.D., not determined.

Acyltransferase	Apparent K_i (μM)		
	Salicylyl-CoA	Benzoyl-CoA	CoA
CAT, forward reaction	7.5 ± 0.5	22 ± 1	22 ± 1
CAT, reverse reaction	9.5 ± 0.5	17 ± 3	–
COT, forward reaction	261 ± 2	295 ± 15	44
COT, reverse reaction	No I	No I	–
CPT-II, forward reaction	> 300	> 300	N.D.

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