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SELECTIVE INHIBITION OF ACYL-CoA DEHYDROGENASES BY A METABOLITE OF HYPOGLYCIN

ECCLESTON A. KEAN

Department of Biochemistry, University of the West Indies, Kingston 7 (Jamaica)

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Summary

Extracts of liver mitochondria from donor rats given hypoglycin, the toxic amino acid from the ackee plant (*Blighia sapida*) showed drastically reduced levels of acyl-CoA dehydrogenase activity with butyryl-CoA as substrate. Activity with octanoyl- and palmitoyl-CoA was unaffected.

Evidence that the active agent is methylenecyclopropylacetyl-CoA, a hypoglycin metabolite, was obtained by observing effects of the compound on a partially purified enzyme mixture prepared from rabbit liver. At 13 μM concentration, it strongly inhibited butyryl-CoA dehydrogenase (EC 1.3.99.2) with butyryl-CoA as substrate; it was far less effective with palmitoyl-CoA as substrate for the other similar enzymes present in the preparation.

Unlike normal substrates of the acyl-CoA dehydrogenases, the compound itself, and not a reaction product, is inhibitory. The observed effect is consistent with quite general inhibition of fatty acid β-oxidation by hypoglycin.

Introduction

Inhibition of fatty acid β-oxidation contributes to the toxic effects of hypoglycin (α-L-amino, β-methylenecyclopropylpropionic acid) [1]. This compound, metabolized like branched-chain amino acids, yields the CoA ester of methylenecyclopropylacetic acid [2]. Interaction of this product with the β-oxidation sequence seems likely, but the affected step has not been identified. Von Holt et al. [3] proposed the acyl-CoA dehydrogenase reaction, involvement of an FAD-dependent enzyme specific for long-chain fatty acids being supported by the chain length of the fatty acids whose oxidation was inhibited (C_{10} or longer, but see ref. 4), and by the protection given against hypoglycin toxicity in vivo by riboflavin. Isovaleryl-CoA dehydrogenase, which may be distinct from butyryl-CoA dehydrogenase, appears to be specifically inhibited by a hypoglycin metabolite also thought to be methylenecyclopropylacetyl-CoA [5,6].

The present study utilized mitochondrial extracts derived from hypoglycin-treated animals to demonstrate inhibition of acyl-CoA dehydrogenase activity, and also examined the effect of methylenecyclopropylacetyl-CoA on purified acyl-CoA dehydrogenases.

Procedure, Results and Discussion

Enzyme activity in rat liver mitochondria

Rats starved overnight received an intramuscular dose of 150 mg hypoglycin [7] /kg, and were killed 4 h later. This is an effective, but not fatal level of dosage [8]. Mitochondria were isolated from the livers of these and of starved control animals by a standard procedure [9] and were then extracted with a detergent mixture. The activities of various enzymes in the extracts were measured.

Acyl-CoA dehydrogenase activity was determined according to Hoskins [10], with phenazine methosulphate as primary electron donor to reduce 2,6-dichloro-phenolindophenol.

To monitor extraction of mitochondrial matrix enzymes, glutamate dehydrogenase (EC 1.4.1.2) was assayed.

Enzyme activity found in the extracts (Table I) indicate that recovery of glutamate dehydrogenase from mitochondria was the same for hypoglycin-treated and control animals. Recoveries of acyl-CoA dehydrogenases, similarly located in the matrix, can therefore be validly compared. The data show that hypoglycin produced a drastic reduction (90%) in acyl-CoA dehydrogenase activity on butyryl-CoA, while activity on octanoyl-CoA and palmitoyl-CoA was not affected. A complication is introduced by the fact that the separate assays with the 3 substrates only partially differentiate between the various acyl-CoA dehydrogenases and will be discussed more fully below.

Methylenecyclopropylacetyl-CoA

Inhibition demonstrated by assay in vitro after being established in vivo could be explained by formation of a remarkably stable E-I complex. The affinity which acyl-CoA dehydrogenases have for their substrates is exception-

TABLE I

ACYL-COA AND GLUTAMATE DEHYDROGENASE ACTIVITIES IN EXTRACTS OF LIVER MITOCHONDRIA FOLLOWING HYPOGLYCIN ADMINISTRATION TO RATS

Each preparation of mitochondrial extract was from pooled liver tissue of two animals. Each group mean ± S.E.M. is from duplicate assays of three such preparations.

Substrate	Treatment group (nmol/min per mg protein)	
	Control	Hypoglycin
Butyryl-CoA	22.7 ± 1.3	2.7 ± 0.3*
Octanoyl-CoA	77.4 ± 11.5	83.0 ± 9.6
Palmitoyl-CoA	18.5 ± 1.8	15.8 ± 1.8
Glutamate	81.0 ± 3.3	78.8 ± 4.3

* Significant difference from control value at $P < 0.001$. Other differences from control values not significant at $P < 0.05$.

ally high, and is even higher for their reaction products, which are inhibitory [11]. To test methylenecyclopropylacetyl-CoA in this connection, the compound was prepared by acylation of CoASH, using the mixed anhydride formed from ethyl hydrogen carbonate and the carboxylic acid [12]. The identity and yield of methylenecyclopropylacetyl-CoA (30—35% of the acid used) was established by hydroxamate formation [12] and also by decrease in absorbance, maximal at 230 nm, upon alkaline hydrolysis [13]. Concentration data given for the compound are derived from the average result of these two methods, which agreed within 12%. In assay media, the compound caused measurable non-enzymic reduction of dichlorophenolindophenol; this reaction reached equilibrium rapidly and was in no way influenced by presence of enzyme. This and other evidence (reaction with 5,5′-dithiobis-(2 nitrobenzoic acid) [14]) indicated a content of free thiol estimated at 15% of total CoA present. It is unlikely that the small amount of CoASH in the product, when included in assay mixtures, would have had any effect on acyl-CoA dehydrogenase activity. Such activity has been successfully assayed in the presence of 0.15 mM CoASH [15]; Osmundsen and Sherratt [16] indicated no inhibition of butyryl-CoA dehydrogenase in control mixtures initially containing 0.04 mM CoASH. When tested at concentrations up to 0.15 mM, methylenecyclopropylacetyl-CoA was not a substrate for the acyl-CoA dehydrogenases studied in the present experiments. Dehydrogenase activity in excess of about 0.25 μM/min would have been detected.

Purified acyl-CoA dehydrogenases

Rabbits are highly susceptible to hypoglycin poisoning (see refs. in [1]), and were convenient for medium-scale enzyme isolation. The enzymes were partially purified from rabbit liver mitochondria [17], resulting in acyl-CoA dehydrogenase activity which was stable at 4°C for about 2 weeks. Specific activity was 0.48 and 0.45 units/mg protein for butyryl- and palmitoyl-CoA respectively (75 μM substrate concentration). About twice this level of activity was found for octanoyl-CoA. A relatively weak activity towards isovaleryl-CoA was lost within 2—3 days. The specific activity data are of limited value for comparison with those of pure enzymes, however, because of the overlapping specificities present in the crude mixture.

There are three distinct mammalian acyl-CoA dehydrogenases [11,18] which could be present in the mixture. A green enzyme, butyryl-CoA dehydrogenase (EC 1.3.99.2), range C_4—C_8, is not active on palmitoyl-CoA. A yellow enzyme, (octanoyl-CoA dehydrogenase [19], EC 1.3.99.3) is active in the range C_4—C_{16}. A second yellow enzyme, palmitoyl-CoA dehydrogenase [20], has the range C_6—$C_{>16}$.

Action of methylenecyclopropylacetyl-CoA on purified enzymes

Kinetic analyses by the Lineweaver-Burk method (Fig. 1, A and B) showed that methylenecyclopropylacetyl-CoA inhibits the action of the enzyme butyryl-CoA dehydrogenase on butyryl-CoA, and that octanoyl-CoA dehydrogenase may be inhibited with butyryl-CoA as substrate, but only marginally, if at all, with palmitoyl-CoA. The plot which resulted with butyryl-CoA as variable substrate (Fig. 1A) is of the hyperbolic type expected [21] when two enzymes,

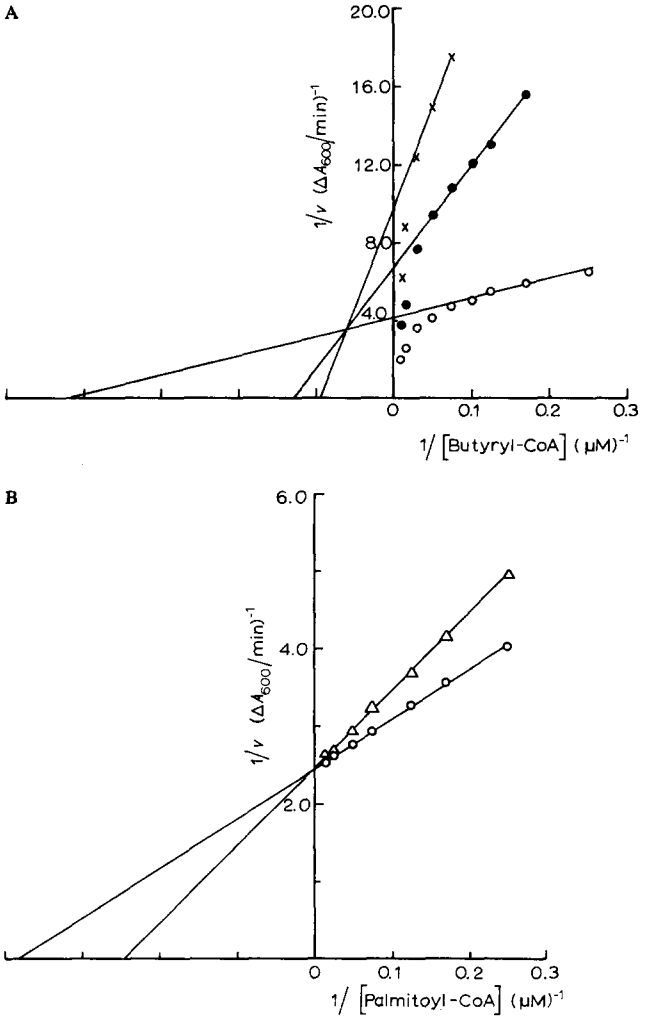

Fig. 1. Inhibition by a hypoglycin metabolite of acyl-CoA dehydrogenase activity in enzyme mixture from rabbit liver mitochondria. Double reciprocal plots for initial rates of dichlorophenolindophenol reduction (ΔA_{600nm}) with (A) butyryl-CoA, (B) palmitoyl-CoA concentrations varied. Concentrations of methylenecyclopropylacetyl-CoA were (μM): ○———○, 0; ●———●, 12.9; X———X, 17.2; △———△, 26. In (A), points obviously on a curve were ignored in fitting straight lines (see text) by the least squares method.

(or two independent active centres in one enzyme), act on a single substrate. Over a range of low [*S*], there is a linear portion where mainly one enzyme species with a high substrate affinity is measured. Strong inhibition of this activity at concentrations of 12.9 and 17.2 μM methylenecyclopropylacetyl-CoA is evident. Inhibition is indicated as a mixture of competitive and non-competitive types, but the true pattern is necessarily obscured by the presence of more than one enzyme activity. Downward inflection of the plot at high [*S*] indicates that the additional enzyme with low substrate affinity becomes increasingly active. The high and low affinity activities most probably belong to butyryl-CoA and octanoyl-CoA dehydrogenase respectively, for which typical

K_m values (pig liver enzymes) are 2.2 and 40 μM butyryl-CoA [18]. A K_m value of 2.4 μM butyryl-CoA for butyryl-CoA dehydrogenase (rabbit liver) is obtained by extrapolation of the linear plot. This ignores the activity contributed at low [*S*] by the high K_m enzyme and therefore indicates a maximum probable value only.

The data of Fig. 1A do not conclusively determine whether the hypoglycin derivative inhibits the action of the high K_m enzyme on butyryl-CoA, although the plots do tend towards the orthodox competitive type pattern at high [*S*]. It is indicated that at [*S*] = 75 μm, this enzyme should be active. At this substrate concentration, there was barely detectable activity on butyryl-CoA in mitochondrial extracts from hypoglycin-treated animals (Table I), while extracts from control rats were found to require addition of about 26 μM inhibitor to the assay media to be reduced to a similarly low level of activity. This could indicate that presence of methylenecyclopropylacetyl-CoA at sufficiently high concentration severely inhibits not only butyryl-CoA dehydrogenase but octanoyl-CoA dehydrogenase as well, as regards activity on butyryl-CoA.

In Fig. 1B, weak competitive inhibition of enzyme activity on palmitoyl-CoA by 26 μM methylenecyclopropylacetyl-CoA is evident. At a level of 17.2 μM, the compound produced barely detectable inhibition (data not shown). As in Fig. 1A, the plot most probably reflects joint activity of two enzymes, in this case octanoyl-CoA and palmitoyl-CoA dehydrogenases. Absence of an inflection implies similarity of their respective K_m values (3.4 and 1.6 μM palmitoyl-CoA for the beef liver enzymes, according to [18]). From Fig. 1B, K_m of about 2.6 μM is obtained for each of the two presumed rabbit liver enzymes. It is undetermined whether the inhibition applies to one enzyme or to both.

The hypothesis that the hypoglycin metabolite methylenecyclopropylacetyl-CoA is the firmly bound inhibitor which can affect butyryl-CoA dehydrogenase and possibly, octanoyl-CoA dehydrogenase activity on butyryl-CoA in vivo receives strong support from the demonstrated effect in vitro. It is interesting that the compound itself is inhibitory without undergoing enzymic transformation. The compound's structure would have been thought to permit α,β-dehydrogenation (compare isobutyryl- and 2-methylbutyryl-CoA, which are attacked [11]). The methylenecyclopropyl grouping therefore appears to be implicated in steric effects at the active sites of these enzymes. This is the first positive identification of a naturally-occurring metabolite other than reaction products of the enzyme active against acyl-CoA dehydrogenases.

Results which complement and support the present findings have been reported elsewhere [16,22]. In that work, incubation of rat liver mitochondria with methylenecyclopropylacetic acid or with methylenecyclopropylpyruvic acid (the α-keto analog of hypoglycin), led to decreased oxidation of palmitoyl -L-carnitine; extracts from such mitochondria showed decreased butyryl-CoA and isovaleryl-CoA dehydrogenase activities, while palmitoyl-CoA dehydrogenase activity was unaffected.

Thus, the inhibitory action appears to be chain-length specific, but in a different sense from the original proposal [3]. Short-chain (C_4), more so than intermediate (C_8) and long-chain (C_{16}) CoA ester, is blocked from reaction with appropriate acyl-CoA dehydrogenase(s), the degree of inhibition presum-

ably being determined by the relative values of K_m and K_i in each particular case. The findings imply that in appropriate circumstances, the commonly used criteria (O_2 consumption, CO_2 or ketone-body production) may indicate that hypoglycin decreases β-oxidation in the case of all fatty acids, whatever their chain length, since these undergo progressive shortening in the process. Also, short-chain fatty acids might be expected to accumulate. Two reports provide notable confirmation. Butyrate oxidation, assessed by $^{14}CO_2$ output from its carboxyl label, was found to be decreased in hypoglycin-treated rats [4], and in such animals, a 14-fold and 2-fold increase in serum levels of butyric acid and hexanoic acid respectively, was found [5]. Such findings, inadequately explained by the earlier hypothesis that long-chain fatty acid oxidation is preferentially inhibited by the toxin, are quite consistent with the conclusions of the present study.

Experimental

Extraction of rat liver mitochondria for enzyme assay

The suspension of mitochondria in 2 ml of 0.25 M sucrose - 1 mM EDTA mixture per g liver tissue was diluted with 0.11 vol. of a mixture containing 10% (v/v) Triton X-100, 0.1 M KH_2PO_4 (pH 7.4), 1 mM dithiothreitol. After stirring 15 min at 4°, centrifugation at 90 000 × *g* for 90 min produced a clear supernatant containing the enzymes which were assayed.

Purified acyl-CoA dehydrogenases

Early steps from [17] were modified as follows. 4.7 g acetone-dried liver mitochondria from normal rabbits were extracted with 80 ml of a 0.1 M KH_2PO_4 (pH 7.4)/3 mM dithiothreitol mixture. After centrifugation, the mixture was treated with 0.1 mg protamine sulphate per mg protein content, and a precipitate discarded. Protein precipitating between 40—60% saturation with $(NH_4)_2SO_4$ was dissolved in 20 mM $NaHCO_3$, yielding a greenish-yellow solution.

Enzyme assays

Acyl-CoA dehydrogenase activity was measured at 30°C, in 20 mM KH_2PO_4 buffer, pH 7.2. A fixed concentration of 33 μM phenazine methosulphate was used [10,23]. Substrate concentration was 75 μM in the standard assay, or varied as shown in the reciprocal plots. The initial rate of dichlorophenol-indophenol reduction was measured at 600 nm in the enzymic reaction initiated by addition of appropriate acyl-CoA substrate to otherwise complete reaction mixtures. Assay of extracts from rat liver mitochondria required about 0.12 mg protein/ml medium; of purified enzymes, about 0.05 mg/ml.

Glutamate dehydrogenase assay was adapted from Strecker [24]. Increase in NADH concentration as glutamate was oxidized was measured as increase in fluorescence (excitation at 360 nm, emission at 470 nm). Approximately 5 μg enzyme protein/ml was used. Media contained 0.8 mM ADP which achieved activation of the enzyme to similar levels in both fresh extracts and extracts stored 16—20 h at 4°C.

Protein was determined by the biuret procedure using bovine serum albumin (Fraction V) as the standard.

Preparation of methylenecyclopropylacetic acid

Hypoglycin was reacted with ninhydrin as in [2], thereafter the procedure differed. The resulting aldehyde was steam-distilled into water. Tollen's ammoniacal silver nitrate [25], which does not attack olefinic linkages, was used as oxidant. The distillate was treated with the amount of reagent calculated to be equivalent, on the basis of Ag content, to twice the amount of amino acid used. After 15 min of stirring, the mixture was filtered and methylenecyclopropylacetic acid in the solution obtained in the eluate from a column of Dowex 50 (H^+ form) ion-exchange resin. A 30-fold excess — relative to hypoglycin used — of cation-exchange capacity was required. The free carboxylic acid was extracted into ether for preparation of the mixed anhydride. The authenticity and purity of methylenecyclopropylacetic acid was confirmed by NMR spectra, and by determination of the neutralization equivalent of the acid generated from a known weight of sodium salt. Overall yield, calculated from weight of the dried sodium salt, was 72%.

Acyl-CoA substrates were obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin, U.S.A. Other biochemicals were from Sigma Chemical Co., St. Louis, Mo., U.S.A.

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