INHIBITION OF MITOCHONDRIAL TRICARBOXYLATE ANION TRANSLOCATION AND OF LIVER FATTY ACID SYNTHESIS BY A NEW HYPOLIPIDEMIC AGENT

Gilles RIBEREAU-GAYON

Centre de Recherche Merrell International 16, rue d'Ankara, 67000-Strasbourg, France

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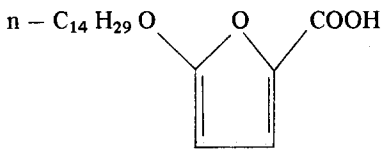
1. Introduction

The translocation of citrate or isocitrate from mitochondria to the cytosol is an obligatory step for fatty acid synthesis from carbohydrates [1–5]. Low concentrations (20–100 μM) of palmityl-CoA have been shown to inhibit citrate translocation across membranes of isolated mitochondria [6]. It has therefore been suggested that long-chain fatty-acyl CoA derivatives are physiological regulators of fatty acid synthesis in liver. No drug with similar properties at such concentrations has been reported. The classical inhibitor of citrate transport, benzene-1,2,3-tricarboxylate, is active in mitochondrial suspensions at 5–10 mM only [5]. Moreover, I have observed (unpublished data) that, when administered to rats, benzene-1,2,3-tricarboxylate has no or only slight action on liver fatty acid synthesis, presumably because sufficiently high cellular concentrations cannot be achieved in vivo.

In this communication, it is shown that 5-(tetradecyloxy)-2-furoic acid (compound I), a hypolipidemic agent which reduces fatty acid biosynthesis in vivo [7], inhibits at low concentrations tricarboxylate anion translocation in membranes of isolated mitochondria. When administered orally to animals, liver fatty acid synthesis from [^{14}C] alanine is inhibited. A direct relationship between the effects of I and two related compounds on citrate translocation and on liver fatty acid synthesis is reported.

2. Materials and methods

The structure of I is as follows:



5-(tetradecyloxy)-2-furoic acid

Two related compounds used in this work were: 4-(tetradecyloxy) benzoic acid (II) and 3-[4-(tetradecyloxy) benzoyl] acetic acid, benzyl ester (III). These three compounds were obtained from Merrell-National Laboratories, Cincinnati, Ohio. The Merrell designation is RMI 14,514 for I, RMI 13,640 for II and RMI 14,425 for III. These three compounds are members of a new class of drugs which produce hypolipidemic effects in rats ([7] and Belgian patents 813 040 for I, 805 172 for II and 805 170 for III). A fourth hypolipidemic drug, Clofibrate (ethyl *p*-chlorophenoxyisobutyrate), was a gift from Dr J. Walker, ICI, Macclesfield, Cheshire, England.

Liver mitochondria were prepared by conventional methods [8] from Sprague-Dawley male rats, 200–250 g, (Charles River, France) fed ad libitum on a commercial diet (UAR, France) or from rats fed a fat-free diet [9] as described for in vivo studies. Mitochondrial protein content was determined by the method of Lowry et al. [10]. The isocitrate influx

into isolated mitochondria was monitored by the rate of intramitochondrial NADH formation in response to the oxidation of externally added *threo*-Ds-isocitrate by mitochondrial NAD-isocitrate dehydrogenase, the conditions being as described by Chappell [11]. Isocitrate was used instead of citrate to bypass the aconitase step, whose activity could be modified by the drugs under study. Moreover, citrate and isocitrate cross mitochondrial membrane by similar mechanisms [3]. Efflux of [^{14}C]citrate from preloaded mitochondria was followed as described by Robinson et al. [12] and Halperin et al. [6].

For the studies of in vivo synthesis of fatty acids, rats (120–130 g) were treated, to increase the synthesis of fatty acids in liver, by a 48 h fast followed by the feeding of a fat-free diet (UAR, France) for 3 h a day during 7 to 11 days. After this period, one or another of the above drugs was added to the same diet (10 g per day) in order to provide a dose of 200 mg/kg/day. Non-anaesthetized rats were injected in the femoral vein, 2 hours after the last daily meal, with a solution of 0.9% (w/v) NaCl containing α-ketoglutarate and radioactive alanine. Thirty minutes after injection rats were sacrificed and incorporation of label into newly synthesized fatty acids was determined. The procedure has been described by Sullivan et al. [9].

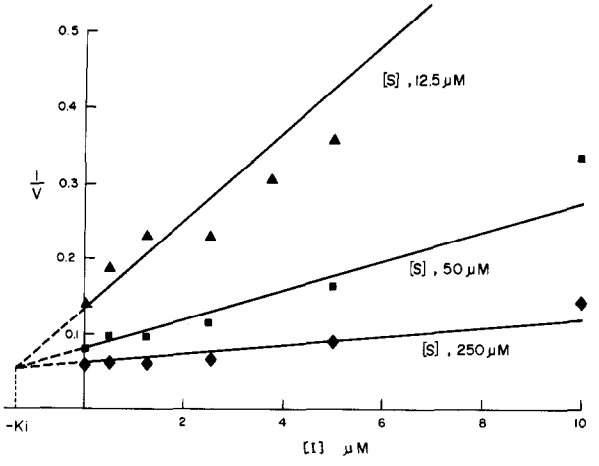


Fig.1. Kinetics of inhibition by compound I of isocitrate influx into liver mitochondria from rats fed a standard diet ad libitum. Rat liver mitochondria (2 mg protein) were suspended in 2.0 ml of a medium containing 125 mM KCl, 20 mM Tris-HCl and 2 mM phosphate, pH 7.4 at 30°C. 1 μM carbonyl cyanide, *m*-chlorophenyl-hydrazone was added, and after 3 min 0.2 μg/ml antimycin, followed by I and 0.25 mM malate. The rate of intramitochondrial NAD reduction, upon the addition of *threo*-Ds-isocitrate at various concentrations, was monitored fluorometrically (excitation 340 nm, emission 460 nm). The reciprocal of the velocity of intra-mitochondrial NAD reduction is plotted against concentration of the inhibitor under three sets of conditions, as indicated in the figure. [S]: *threo*-Ds-isocitrate. The K_i is 1.4 μM.

3. Results

The kinetics of inhibition by I of isocitrate influx into mitochondria of rats fed a standard diet is shown in fig.1. A competitive inhibition was observed. The apparent K_i was 1.4 μM while the apparent K_M for *threo*-Ds-isocitrate was 23 μM. For rats on a fat-free diet the values were 2.1 μM and 26 μM respectively. Inhibition was identical whether I was added before or after the anti-porter anion, malate. Inhibition of isocitrate influx by 4 μM I was not altered when various malate concentrations, from 25 μM to 1 mM, were used. No inhibition of NAD-isocitrate dehydrogenase from disrupted mitochondria was found with 20 μM I while, under the same conditions in intact mitochondria, isocitrate oxidation was inhibited 85%. Compound I inhibited [^{14}C]citrate efflux from preloaded mitochondria as shown in fig.2 for rats fed a standard diet. The

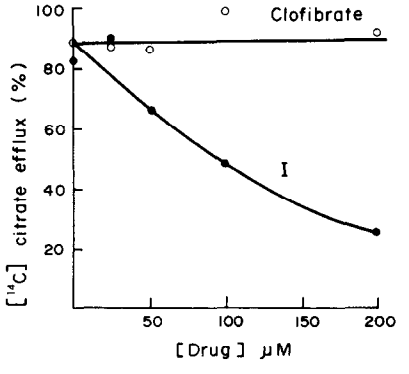


Fig.2. Effects of compound I and clofibrate on the efflux of citrate from mitochondria isolated from rats fed a standard diet ad libitum and preloaded with [^{14}C]citrate. Efflux was initiated by the addition of non-labelled citrate (1 mM). Mitochondrial protein, 8 mg/ml; t = 18°C.

Table 1
Effects of hypolipidemic agents (compounds I, II, III and clofibrate) on net in vivo incorporation of label from [U-^{14}C]alanine into liver fatty acids[c]

Treatment group (N° of animals)		Liver weight (g)	In vivo lipogenesis dpm in fatty acids (mg liver/30 min)
1. Control	(5)	6.2 ± 0.2	17.8 ± 2.3
Compound I	(4)	6.4 ± 0.2	9.6 ± 2.3[b]
Clofibrate	(5)	6.6 ± 0.4	15.4 ± 1.7
2. Control	(5)	7.2 ± 0.4	15.8 ± 1.2
Compound II	(5)	7.2 ± 0.3	11.7 ± 1.1[a]
3. Control	(4)	6.6 ± 0.4	22.2 ± 2.0
Compound III	(4)	7.6 ± 0.7	17.1 ± 2.6

Results are mean ± SEM.
[a] $p < 0.1$,
[b] $p < 0.05$, Student's 2-tailed t-test.
[c] In all cases drugs were added to the diet for 4 days at a dose of 200 mg/kg/day. Diet was fat-free and given 3 h a day. [U-^{14}C]alanine, 80 mg (15 μCi)/kg of body weight, was injected to rats as described in the text.

inhibition was also observed in mitochondria from rats on the fat-free regimen. The hypolipidemic agent clofibrate, which is known to inhibit purified acetyl-CoA carboxylase [13], had no inhibitory activity on citrate efflux.

The effects of I, II, III and clofibrate on the in vivo rate of liver lipogenesis from [^{14}C]alanine are reported in table 1. Compound I was the strongest inhibitor of label incorporation into fatty acids (46%) followed by II (26%) and III (23%). Under the same conditions clofibrate slightly inhibited liver fatty acid synthesis (13%). Using mitochondria isolated from rats on a standard diet, compounds I, II and III, each at a concentration of 25 μM, inhibited influx of *threo*-Ds-isocitrate (25 μM) by 83%, 36% and 27%, respectively. At the same concentration clofibrate inhibited isocitrate influx by 5%.

4. Discussion

It is apparent from these results that:

(1) Compound I, like palmityl-CoA, inhibited translocation of tricarboxylate anion across mitochondrial membrane at low concentrations. This compound being highly lipophilic, such concentrations may be obtained in liver cells after treatment of animals with the drug at doses (100–200 mg/kg) which lower plasma triglycerides and cholesterol.

(2) Compound I inhibited isocitrate influx into isolated mitochondria at lower concentrations than citrate efflux. However, the protein contents for the two assays were different. If active drug is bound to the citrate carrier, a more appropriate comparison is made by expressing concentrations in nmoles/mg of protein [6]. In this case, inhibition was observed at 5–20 nmol/mg of protein for both conditions.

(3) Compound I had a greater affinity than the substrate for the tricarboxylate anion carrier. Indeed, the apparent K_i for I was approx. 10 times smaller than the K_M for *threo*-Ds-isocitrate.

(4) In vivo liver fatty acid synthesis from [^{14}C] alanine was significantly inhibited by I. In alanine metabolism, the pyruvate formed is transported into mitochondria by a specific carrier. Pyruvate dehydrogenase is localized on the inner side of the inner membrane, and therefore fatty acid synthesis from alanine involves citrate formation in mitochondria and its efflux to the cytosol. These in vivo effects of I and those described on isolated mitochondria suggest that pharmacological action of I is mediated through an inhibition of mitochondrial citrate transport. However, it is not excluded that I inhibits also acetyl-CoA carboxylase [14].

(5) There was a direct relationship between the in vivo inhibition of fatty acid synthesis and the inhibition of isocitrate influx by I and two related compounds (II and III). This relationship further substantiates the hypothesis for the mechanism of action of I.

(6) Clofibrate had only slight action on tricarboxylate anion transport and on liver fatty acid synthesis from [^{14}C]alanine indicating that this drug and I have different effects on liver lipogenesis.

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