Evaluation of malonyl-CoA in the regulation of long-chain fatty acid oxidation in the liver

Evidence for an unidentified regulatory component of the system

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(Received 5 September 1980/Accepted 15 September 1980)

Palmitate oxidation by liver mitochondria from fed and starved rats exhibited markedly different sensitivities to inhibition by malonyl-CoA. In the mitochondrial system from fed rats, 50% inhibition required $19\text{ }\mu\text{M}$ -malonyl-CoA, whereas the mitochondria from starved rats were by comparison refractory to malonyl-CoA. Inhibition by malonyl-CoA was completely reversed by increasing the molar ratio of fatty acid to albumin. Results indicate that the potential effectiveness of malonyl-CoA as an inhibitor of fatty acid oxidation in the liver is dependent on an unidentified regulatory component of the system. The functional activity of this component is modified by the nutritional state, and its site of action is at the mitochondrial level.

It has been suggested by McGarry and Foster and co-workers (McGarry *et al.*, 1977, 1978a,c) that malonyl-CoA is involved in the regulation of fatty acid oxidation and ketogenesis in the liver. They have suggested that in the fed dietary state malonyl-CoA produced from carbohydrate inhibits fatty acid oxidation by inhibiting acylcarnitine formation at the site of carnitine acyltransferase I (McGarry *et al.*, 1978b). A K_i of only $1.5\text{ }\mu\text{M}$ -malonyl-CoA was observed. They have concluded (McGarry & Foster, 1979) that full activation of hepatic fatty acid oxidation, as occurs in starvation, can be quantitatively accounted for by depletion of malonyl-CoA and elevation of the liver carnitine concentration. Cook *et al.* (1978) concluded that in hepatocytes from meal-fed rats malonyl-CoA is a determinant of ketogenic activity, but they stated that its significance in ketotic states *in vivo* remains to be determined.

The present study was undertaken to examine the possible regulatory function of malonyl-CoA by analysing the effects of malonyl-CoA on long-chain fatty acid oxidation by rat liver mitochondria as a function of the nutritional state and the molar ratio of fatty acid to albumin. These observations demonstrate that the sensitivity of fatty acid oxidation to malonyl-CoA is dependent on an unidentified regulatory component of the system, which is

operative at the mitochondrial level and modulated by the nutritional state. This investigation has appeared in abstract form (Ontko *et al.*, 1980). The difference in sensitivities of fatty acid oxidation to malonyl-CoA exhibited by liver mitochondria from fed and starved rats, as described herein, has also been observed by Cook *et al.* (1980).

Experimental

Male Sprague–Dawley rats (150–250 g) were given water and Purina Laboratory Chow *ad libitum*. When indicated, rats were fed on a low-fat diet (no. 901682; ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.), which contained (w/w) 58.5% sucrose, 21% casein, 16.5% cellulose, less than 1% fat, vitamins and minerals. When rats were starved, food was removed 24 h before the experiment. Animals were killed by decapitation. Livers were placed in ice-cold 0.25 M -sucrose/ 3 mM -Tris/ $-Cl/1\text{ mM}$ -EGTA, pH 7.4, or in 0.25 M -sucrose alone, as indicated. All processing steps were at $0\text{--}3^\circ\text{C}$. After slicing, livers were homogenized with a loose-fitting Potter–Elvehjem homogenizer (Otto & Ontko, 1978). The mitochondrial fraction was isolated in the sucrose/Tris/EGTA medium, or in sucrose alone when indicated, and suspended in 150 mM -KCl as described by McGarry *et al.*

(1978b), and kept at 0–3°C. Mitochondria exhibited respiratory control ratios (Estabrook, 1967) of 4.0–7.0 with 5 mM-glutamate plus 5 mM-malate as substrates.

Mitochondria were incubated in the modified Krebs–Henseleit medium described by McGarry *et al.* (1977). It contained 4.9 mM-NaCl, 25.5 mM-NaHCO₃, 121 mM-KCl, 2.4 mM-MgSO₄ and 1.2 mM-KH₂PO₄ and was equilibrated with O₂/CO₂ (19:1). Final concentrations of the following were included: 4 mM-ATP, 100 μ M-L-carnitine, 50 μ M-CoA, 40 μ M-sodium palmitate and 0.8% bovine serum albumin. The sub-saturating concentration of 40 μ M-palmitate was employed in order to duplicate the experimental conditions of McGarry *et al.* (1978b). The incubation medium was prepared by adding ATP, carnitine, CoA and palmitate/albumin in a combined volume of 0.75 ml at pH 7.4 to 1.25 ml of double-strength modified Krebs–Henseleit medium. The malonyl-CoA at pH 7.4 was next added. Mitochondria (0.5 ml in 150 mM-KCl) were then added, flasks were gassed with O₂/CO₂ (19:1), capped, and the final system (2.5 ml, pH 7.4) was incubated for 10 min at 30°C in a Dubnoff water bath at 80 oscillations/min.

Reactions were terminated with 0.5 ml of 30% (w/v) HClO₄. Supernatants were adjusted to pH 6.5–7.0 with 3 M-K₂CO₃ for analyses of acetoacetate and β -hydroxybutyrate (Williamson *et al.*, 1962). Only small amounts of β -hydroxybutyrate were present. These were routinely analysed and included with the measured acetoacetate. Under the conditions employed, palmitate was almost exclusively oxidized to ketone bodies, which therefore provided accurate estimation of palmitate oxidation. Esterification of palmitate to phospholipids and neutral lipids was almost nil under these conditions.

Chemicals were obtained from the sources previously indicated (Otto & Ontko, 1978). Malonyl-CoA was obtained from P-L Laboratories, Milwaukee, WI, U.S.A. This product exhibited the correct absorption ratios ($A_{232}/A_{260} = 0.56$; $A_{250}/A_{260} = 0.95$; $A_{280}/A_{260} = 0.22$) measured at pH 2. Malonyl-CoA solutions were prepared each day of the experiment by dissolving in 5 mM-potassium phosphate, pH 7.4, and re-adjusting to pH 7.4. Additions of malonyl-CoA to various flasks were equalized in volume with 5 mM-phosphate, which was thereby increased by only 0.06 mM in the system.

Results and discussion

If malonyl-CoA regulates the β -oxidation of fatty acids in the liver, via inhibition of acylcarnitine formation, it should inhibit the oxidation of fatty acids by isolated liver mitochondria. The only direct evidence for this action of malonyl-CoA was

provided by McGarry *et al.* (1978b); they reported that malonyl-CoA decreased the production of labelled ketone bodies from [1-¹⁴C]palmitate by liver mitochondria.

We have examined the effects of malonyl-CoA on ketone-body production from palmitate by mitochondria from fed and starved rats by direct enzymic determinations of acetoacetate and β -hydroxybutyrate accumulation. The incubation system described by McGarry *et al.* (1978b) was employed. Palmitate oxidation by mitochondria from fed rats exhibited significantly greater sensitivity to malonyl-CoA than did fatty acid oxidation by mitochondria derived from starved animals (Fig. 1); 50% inhibition required 19 μ M-malonyl-CoA, 10 times higher than observed by McGarry *et al.* (1978b). We have no explanation for this difference. The concentration of malonyl-CoA in liver cells from fed and starved rats appears to be in the range

Fig. 1. Sensitivities of liver mitochondrial palmitate oxidation to malonyl-CoA

Liver mitochondria (4.7–7.5 mg of protein) from fed (●) and 24 h-starved (○) rats were incubated for 10 min at 30°C as described in the Experimental section. Six separate malonyl-CoA titrations of total ketone-body production were conducted, each with mitochondria isolated from a different rat (three fed and three starved). The average control rates of ketogenesis were 4.82 and 4.75 nmol/min per mg of protein (fed and starved respectively). Values shown are the means for three, $\pm$ S.E.M. Values for fed and starved rats were analysed by Student's *t* test; $P < 0.01$ at 10 and 15 μ M-malonyl-CoA, $P < 0.05$ at 20 and 30 μ M-malonyl-CoA.

1–6 μM (Cook *et al.*, 1978; McGarry *et al.*, 1978c; McGarry & Foster, 1979). Effective participation of malonyl-CoA in the regulation of fatty acid oxidation in the liver requires somewhat greater inhibitory potency than was observed in the current studies (Fig. 1). McGarry *et al.* (1978b) fed their animals on a low-fat diet. We therefore maintained rats on such a diet and repeated the malonyl-CoA titrations of liver mitochondrial palmitate oxidation. Results were identical with those shown in Fig. 1. We then considered that the EGTA in the mitochondrial isolation medium may have depleted endogenous Ca^{2+} and produced a malonyl-CoA sensitivity different from that observed with mitochondria isolated from sucrose alone as used by McGarry *et al.* (1978b). Repetition of the malonyl-CoA titrations with mitochondria isolated in 0.25 M-sucrose alone provided results indistinguishable from those of Fig. 1.

McGarry *et al.* (1977) noted that oleate oxidation in liver homogenates from starved rats was somewhat less sensitive to malonyl-CoA (200 μM) than in homogenates of liver from fed rats. The difference was 68% inhibition after 24 h starvation and 88% in the fed condition. It is possible that the difference in malonyl-CoA sensitivity of the mitochondrial fraction, shown in Fig. 1, may have contributed to the difference in the homogenate systems (McGarry *et al.*, 1977). However, the high concentration of malonyl-CoA employed (200 μM), the complexity of the homogenate system and the relatively small difference observed by McGarry *et al.* (1977) do not suggest a metabolic significance. Thus, such a difference might have been caused by differential rates of malonyl-CoA formation and/or utilization in the two homogenate systems. The present experiments clearly demonstrate a difference and eliminate an absolute requirement for cytosolic factors and processes for the operation of altered malonyl-CoA sensitivity at the mitochondrial level. Cytosolic factors may, however, be involved in the development of this change. Also, it remains possible that cytosolic factors interact with mitochondrial sites of malonyl-CoA sensitivity and further modify this activity.

We employed a molar ratio of palmitate to albumin of 0.34, as used by McGarry *et al.* (1978b). At the rat plasma albumin concentration of 3.3% (w/v) (Peters & Peters, 1972), the ratio employed is equivalent to a plasma non-esterified fatty acid concentration of 165 μM , considerably below physiological conditions. In the normal fed state the ratio is 0.6–0.7. Therefore the effect of the molar ratio on the inhibition of fatty acid oxidation by malonyl-CoA was examined (Fig. 2): 20 μM -malonyl-CoA, near the K_i value of 19 μM (Fig. 1), was employed. As the palmitate/albumin ratio was elevated, the inhibitory effect of malonyl-CoA was clearly rever-

Fig. 2. Effect of palmitate/albumin molar ratio on inhibition of palmitate oxidation by malonyl-CoA. Liver mitochondria (4.4–5.1 mg of protein) from fed rats were incubated as described in Fig. 1. Total ketone-body production in the absence and presence of 20 μM -malonyl-CoA was measured in three separate experiments, each with molar ratios 0.34, 0.52, 0.90, 1.29 and 3.45. The concentration of albumin was 0.8% in all systems. Values shown are the means for three, $\pm$ S.E.M.

sed. Although intracellular concentrations, protein binding and compartmentation of non-esterified fatty acids are unclear, it is generally regarded that intracellular concentrations respond to alterations in the plasma (Dietschy, 1978). The data of Fig. 2 suggest that oscillations in plasma non-esterified fatty acids, which occur in interconversions between fed and starved states, influence the potential inhibitory effectiveness of malonyl-CoA on long-chain fatty acid oxidation. It should also be noted that the palmitate/albumin ratio declines during incubations as the fatty acid is utilized. Thus, low experimental molar ratios of non-esterified fatty acid to albumin, in the presence of excess ATP and CoA, limit the acyl-CoA concentration and may accentuate the potency of malonyl-CoA as a competitive inhibitor of acylcarnitine formation.

Increased concentrations of non-esterified fatty acids elevate fatty acid oxidation via effective increase in substrate alone. A second mechanism by which non-esterified fatty acids may stimulate their oxidative metabolism is via decrease in cellular malonyl-CoA (Cook *et al.*, 1978). The present study suggests a third mechanism, namely decreased inhibitory efficiency of malonyl-CoA (Fig. 2),

presumably because of an increase in the concentration of the natural substrate, acyl-CoA, in the synthesis of acylcarnitine.

Snoswell & Koundakjian (1972) observed increased liver carnitine in diabetic rats. Starvation was also observed to increase liver carnitine (Kondrup & Grunnet, 1973). McGarry *et al.* (1975) proposed that this increase participates in the regulation of ketogenesis. Parvin & Pande (1979) suggested enhancement of fatty acid transport via carnitine/acylcarnitine translocase (Pande, 1975; Ramsey & Tubbs, 1975) in ketogenic states via increased intramitochondrial carnitine. We considered the possibility that the relative resistance of the liver mitochondria from starved rats to malonyl-CoA (Fig. 1) was a function of endogenous carnitine. Thus, malonyl-CoA-induced inhibition of palmitoylcarnitine formation, resulting in decreased palmitoylcarnitine translocation, might be counteracted by mitochondrial-matrix-carnitine-induced stimulation of exchange diffusion. However, Pande & Parvin (1980) have observed that in liver mitochondria unidirectional transport of free carnitine causes its rapid equilibration across the inner membrane. In the present study equilibration was probably achieved in 20 s at 30°C. The studies of Parvin & Pande (1980) therefore eliminate the carnitine concentration as a causative factor in the relative resistance of mitochondria from starved rats to inhibition by malonyl-CoA (Fig. 1).

The present results add an order of complexity to the regulation of fatty acid oxidation in the liver and indicate that the concentrations of malonyl-CoA and carnitine, previously concluded to account quantitatively for the changes in fatty acid oxidation produced by starvation (McGarry & Foster, 1979), do not fully explain the rate-control of the process. An unidentified component of the multireaction system, itself regulated by the nutritional state, appears to determine the inhibitory effectiveness of malonyl-CoA. The nature of this interaction and the events involved in its expression are unknown. The relative contributions of these factors to the intracellular regulation of fatty acid oxidation remain to be determined.

The technical assistance of T. Lanell Tompkins is gratefully acknowledged. This investigation was supported by Grant HL 23181 from the U.S. Public Health Service.

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