

Differential inhibition of ketogenesis by malonyl-CoA in mitochondria from fed and starved rats

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Rates of ketogenesis in mitochondria from fed or starved rats were identical at optimal substrate concentrations, but responded differently to inhibition by malonyl-CoA. Kinetic data suggest that the K_i for malonyl-CoA is greater in the starved animal. These results indicate that, for the regulation of ketogenesis in the starved state, the lower sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA may be more important than the concentration of malonyl-CoA.

States of rapid ketogenesis such as starvation and diabetes are associated with high concentrations of plasma non-esterified fatty acids. However, even when the amounts of fatty acids supplied to the liver are equal, starved rats produce ketone bodies at a higher rate than in fed rats (Rubenstein & Rubenstein, 1966; Mayes, 1970; Ontko, 1972; McGarry *et al.*, 1973). More recent evidence suggests that the oxidation of fatty acids may be inhibited by malonyl-CoA at the carnitine palmitoyltransferase I reaction, an inhibition that is competitive with acyl-CoA (McGarry *et al.*, 1977, 1978*a,b*; Cook *et al.*, 1978; McGarry & Foster, 1979). It has been proposed that the higher liver content of malonyl-CoA in fed rats explains the difference in ketogenic rates between fed and starved animals (McGarry *et al.*, 1978*a,b*; McGarry & Foster, 1979). However, most of the evidence supporting a role for malonyl-CoA in the regulation of ketogenesis has come from experiments with isolated hepatocytes (Cook *et al.*, 1978; McGarry *et al.*, 1978*a,b*; McGarry & Foster, 1979), liver homogenates (McGarry *et al.*, 1977, 1978*a*; Cook *et al.*, 1978) or isolated liver mitochondria (McGarry *et al.*, 1978*b*) derived from fed animals. Our previous experiments with hepatocytes isolated from meal-fed rats suggested that rates of ketogenesis were inversely correlated with the hepatocyte malonyl-CoA content (Cook *et al.*, 1978); however, there was no such correlation in hepatocytes from starved animals (Cook & Veech, 1978). Furthermore, Benito & Williamson (1978) have suggested that lowering malonyl-CoA concentrations is not sufficient to explain the increased ketogenesis in starvation (see also Whitelaw & Williamson, 1977). Experiments presented here indicate that malonyl-CoA does not play the same role in starved animals that it does in fed animals.

Similar conclusions have been made independently in another laboratory (Ontko *et al.*, 1980; Ontko & Johns, 1980).

Experimental

Methods

Male Wistar rats weighing 200–250 g at the time of use were meal-fed with the standard NIH rat diet (5% fat, 24% protein, 54% carbohydrate, all w/w) between 08:00 and 11:00 h for at least 6 days before being used (meal-fed rats) or starved for 48 h after meal-feeding as above (starved rats). Both groups of rats had free access to water.

Mitochondria were isolated from rat liver homogenates as described by Otto & Ontko (1978), except that EGTA was replaced by EDTA in the isolation medium. All loosely packed mitochondria at the top of the pellet were discarded after the last 9000 g centrifugation. The final pellet was resuspended in 0.25 M-sucrose/3 mM-Tris/HCl (pH 7.4 at 4°C) to provide a concentration of 20 mg of mitochondrial protein/ml. All mitochondrial preparations in these experiments had a respiratory control ratio (Estabrook, 1967) above 6.0 with the substrates 5 mM-glutamate plus 5 mM-malate.

The mitochondrial suspension (0.25 ml) was incubated with 0.5 ml of 0.25 M-sucrose/3 mM-Tris/HCl, 0.5 ml of 1.8 mM-palmitate/6% (w/v) bovine albumin (dialysed fatty acid free) and 0.75 ml of mixed medium (pH 7.0–7.1) to provide final concentrations of 60 mM-KCl, 10 mM-potassium phosphate, 5 mM-MgCl₂, 3 mM-ATP, 0.8 mM-DL-carnitine, 20 μM-CoA and 1 mM-dithiothreitol in 2 ml final volume. These concentrations gave optimum ketogenic rates in mitochondria from both fed and starved rats. Incubations were carried out at 37°C.

Rates of ketone-body production were optimal and linear with respect to time after 2 min, and the ketogenic rates presented here were measured between 2 and 4 min. There was no net increase in ketone bodies from endogenous substrates during this time. In mitochondrial preparations isolated as described here, there was negligible esterification of added fatty acid (D. A. Otto, unpublished work).

For analyses of ketone bodies and malonyl-CoA, the incubations were terminated with HClO_4 as described by Otto & Ontko (1978). Malonyl-CoA (Cook *et al.*, 1978; Guynn *et al.*, 1972), acetoacetate (Mellanby & Williamson, 1974) and 3-hydroxybutyrate (Williamson & Mellanby, 1974) were determined enzymically in neutralized HClO_4 extracts. Protein was measured as described by Lowry *et al.* (1951), as modified by Markwell *et al.* (1978).

Materials

Fatty acid synthetase used in the malonyl-CoA assay was generously provided by Dr. Tracy Linn (V. A. Hospital, Preclinical Science Unit, Dallas, TX, U.S.A.). Rats were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). ATP, NADH, NADPH, NAD^+ , DL-carnitine, dithiothreitol, EDTA, Tris (Trizma base) and palmitic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pentex bovine albumin (Fraction V; fatty acid-free) was a product of Miles Laboratories (Elkhart, IN, U.S.A.) and was dialysed against 0.9% NaCl, followed by distilled water, before being used. CoA, acetyl-CoA and malonyl-CoA were supplied by P-L Biochemicals (Milwaukee, WI, U.S.A.). Stock malonyl-CoA solutions were assayed enzymically by the method of Guynn *et al.* (1972) and found to conform to the manufacturer's specifications ($94 \pm 5\%$). The concentration determined by this assay was used in calculating the amount of malonyl-CoA added to each incubation mixture. 3-Hydroxybutyrate dehydrogenase was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). All other chemicals were reagent-grade products from commercial sources.

Results and discussion

We have used the term ketogenesis in this paper in a general sense that indicates the production of ketone bodies by isolated mitochondria from added palmitate. Ketogenesis in this sense refers to the entire process, beginning with the activation of fatty acids and leading to the eventual production of acetoacetate and 3-hydroxybutyrate from acetyl-CoA. Under the conditions employed in our experiments, the production of $^{14}\text{CO}_2$ from ^{14}C -labelled fatty acids accounted for no more than 10% of the

fatty acid oxidized, and thus ketone-body production can be used as an accurate index of the rate of the overall process (see also Otto & Ontko, 1978; Lopes-Cardozo *et al.*, 1975).

Mitochondria isolated from either starved or fed rats showed optimal rates of ketogenesis from added palmitate at the same concentrations of other added substrates (see the Experimental section). Mitochondria from fed and starved rats produced ketone bodies at rates of 15.2 ± 0.5 (mean \pm S.E.M.; $n = 9$) and 15.3 ± 0.8 ($n = 10$) nmol/min per mg of mitochondrial protein respectively. These rates were not affected significantly by peroxisomal fatty acid oxidation (Lazarow & de Duve, 1976; Lazarow, 1978), because omitting carnitine, which is required for mitochondrial oxidation of fatty acids (Fritz, 1961) but not required for peroxisomal fatty acid oxidation (Lazarow & de Duve, 1976), inhibited ketogenesis in three preparations of mitochondria by an average of 91%, and addition of 1 mM-KCN, which blocks fatty acid oxidation in mitochondria (Mannaerts *et al.*, 1979) but has no effect on peroxisomes (Lazarow & de Duve, 1976), resulted in 100% inhibition of ketogenesis.

There was a large difference in the response of ketogenesis to inhibition by malonyl-CoA depending on the source of mitochondria. The mitochondria from starved rats were much less responsive to malonyl-CoA inhibition, and in fact there was no inhibition at all at concentrations of $10 \mu\text{M}$ or less [$10 \mu\text{M}$ -malonyl-CoA produced $33 \pm 5\%$ ($n = 3$) inhibition in mitochondria from fed rats]. Thus, at malonyl-CoA concentrations expected in the liver of starved rats *in vivo* (Guynn *et al.*, 1972), ketogenesis would not be inhibited. Even at very high concentrations (up to $100 \mu\text{M}$), malonyl-CoA was a much less effective inhibitor in mitochondria from starved rats. At $20 \mu\text{M}$ -malonyl-CoA, $57 \pm 4\%$ ($n = 3$) inhibition was produced in mitochondria from fed rats, but mitochondria from starved rats required $100 \mu\text{M}$ -malonyl-CoA to inhibit ketogenesis by $58 \pm 5\%$ ($n = 3$).

Rat liver is reported to contain an enzyme that deacylates malonyl-CoA to produce free CoA (McGarry *et al.*, 1978b). If there were more deacylase present in mitochondria from starved rats than in those from fed rats, effective malonyl-CoA concentrations could be altered. Measuring malonyl-CoA at the beginning and end of the 2 min incubation period indicated no more than a 10% loss of malonyl-CoA during incubation. These as well as additional experiments carried out for longer time periods showed that the rate of loss of malonyl-CoA from the incubation medium was equal in preparations from fed or starved rats. We have also observed that once the malonyl-CoA has been removed, ketogenic rates returned to the uninhibited rates. Thus malonyl-CoA inhibition is reversible.

These mitochondrial experiments suggest that it is not the malonyl-CoA content of liver alone that is important in malonyl-CoA inhibition of ketogenesis, but that the responsiveness of liver to the action of malonyl-CoA plays a primary role.

Fig. 1 contains the results of mitochondrial incubations in which malonyl-CoA concentration was varied. These data are plotted as proposed by Rognstad (1979), whereby the shape of the plot gives some information about the activity of the inhibited enzyme relative to the overall rate of the multienzyme process. Since malonyl-CoA is a competitive inhibitor of carnitine palmitoyltransferase (McGarry *et al.*, 1977, 1978a,b; Cook *et al.*, 1978; McGarry & Foster, 1979), its effect is to increase the K_m for substrate. Therefore, if the transferase reaction itself or a reaction that precedes it were rate-limiting for the pathway, ketogenesis should be inhibited at all concentrations of malonyl-CoA. The absence of any effect of malonyl-CoA at low concentrations indicates that carnitine palmitoyltransferase is present in excess and that some reaction after it is rate-limiting for ketone-body production by mitochondria from both fed (Fig. 1a) and starved (Fig. 1b) rats. However, fed rats *in vivo* would possess hepatic contents of malonyl-CoA great enough to make this enzyme rate-limiting. The slope and intercept terms for the lines in Fig. 1 are:

$$\frac{K_m}{V_{\max} \cdot K_i \cdot [S]}, \text{ the slope;}$$

$$\left(\frac{1}{V_{\max}}\right) \cdot \left(1 + \frac{K_m}{[S]}\right), \text{ the extrapolated ordinate intercept;}$$

$$\text{and } -K_i \left(1 + \frac{[S]}{K_m}\right), \text{ the abscissa intercept.}$$

In analysing those terms, it should be noted that with mitochondria from either source maximal rates of ketogenesis were obtained with palmitate concentrations of 0.07 mM and greater, and the experiments described here were conducted with 0.45 mM-palmitate. Furthermore, the acyl-CoA synthetase in our mitochondrial preparations produced palmitoyl-CoA at a rate that was at least five times the rate of palmitoyl-CoA utilization for ketone-body synthesis (G. A. Cook & D. A. Otto, unpublished work). From this we assume that the concentrations of palmitoyl-CoA were saturating and much larger than the K_m of carnitine palmitoyltransferase for this substrate. In that case, both the smaller slope and the more negative abscissa intercept seen with mitochondria from starved rats would be consistent with a larger K_i for malonyl-CoA, and we take as a working hypothesis that starvation results in a modified carnitine palmitoyltransferase or in a change in some factor that makes this enzyme less sensitive to inhibition by malonyl-CoA.

Experiments presented here suggest the following. (a) Malonyl-CoA is much more important in the fed animal than in the starved animal, possibly to prevent futile cycling that might occur by oxidation of newly synthesized fatty acids. (b) Malonyl-CoA need not be completely eliminated to relieve its inhibition of ketogenesis, since starvation induces a mechanism by which the liver becomes less responsive to malonyl-CoA inhibition. Thus optimal rates of ketogenesis could be achieved in starved animals in the presence of malonyl-CoA concentrations that would be inhibitory in the fed animal. (c) Kinetic data suggest that the K_i for malonyl-CoA inhibition of ketogenesis is increased during starvation. (d) Although carnitine palmitoyltransferase is present in excess in mitochondria from both fed and starved rats, malonyl-CoA can make this enzyme rate-

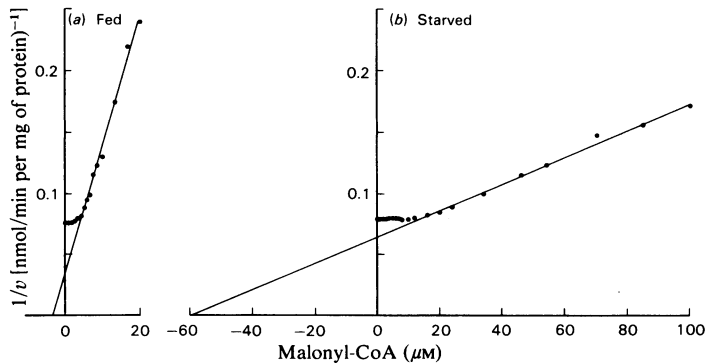


Fig. 1. Inhibition of ketogenesis in mitochondria from (a) meal-fed or (b) 48 h-starved rats

Data from a single experiment (representative of three experiments) are plotted according to Rognstad (1979). Saturating concentrations of palmitate were used.

limiting in the fed state, but in the starved state the rate of ketogenesis must be regulated elsewhere, either within or outside the mitochondria.

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