The flux control coefficient of carnitine palmitoyltransferase I on palmitate β -oxidation in rat hepatocyte cultures

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Two important factors that determine the flux of hepatic β -oxidation of long-chain fatty acids are the availability of fatty acid and the activity of carnitine palmitoyltransferase I (CPT I). Using Metabolic Control Analysis, the flux control coefficient of CPT I in rat hepatocyte monolayers was determined by titration with 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (Etomoxir), which is converted to Etomoxir-CoA, an irreversible inhibitor of CPT I. We measured CPT I activity and flux through β -oxidation at 0.2 mM and 1.0 mM palmitate to simulate

substrate concentrations in fed and fasted states. Rates of β -oxidation were 4.5-fold higher at 1.0 mM palmitate compared with 0.2 mM palmitate. Flux control coefficients of CPT I, estimated by two independent methods, were similar: 0.67 and 0.79 for 0.2 mM palmitate, and 0.68 and 0.77 for 1 mM palmitate. It is concluded that the regulatory potential of CPT I is similar at low and high physiological concentrations of palmitate.

INTRODUCTION

Current understanding of the regulation of fatty acid oxidation and ketogenesis is incomplete [1]. Partition of long-chain acyl-CoA esters between esterification and oxidation is thought to be regulated by carnitine palmitoyltransferase I (CPT I) activity, which is sensitive to inhibition by malonyl-CoA [2]. However, rapid changes in ketogenic flux are not necessarily paralleled by alterations in CPT I activity [3,4]. Alternative enzymes also suggested to play a role include those of the β -oxidation spiral [5–7] or the hydroxymethylglutaryl-CoA pathway [8].

Metabolic Control Analysis (MCA) allows quantitative analysis of the distribution of control in a pathway [9–11]. It has been used previously to measure the distribution of control in the β -oxidation/ketogenic pathways in uncoupled, isolated rat liver mitochondria. In some conditions, up to 72% of the control of ketogenesis was by the enzymes of the hydroxymethylglutaryl-CoA pathway [12,13]. Inhibitor titration of some enzymes involved in the oxidation of palmitoyl-carnitine indicated that control was after CPT II [14]. At low rates of palmitoyl-CoA oxidation CPT I was not rate-controlling, although control shifts to CPT I when flux through β -oxidation increases [15]. More recently, MCA has been applied to palmitoyl-CoA oxidation and ketogenesis in coupled mitochondria isolated from rat liver [16]. In state 3 most of the control through β -oxidation/ketogenesis resided at CPT I, whilst in states intermediate between 3 and 4, control was shared between CPT I and the enzymes of β -oxidation/ketogenesis. However, since CPT I activity is affected by the intracellular environment [17], studies on isolated mitochondria may not reflect the control exerted in the intact cell. Recent work on freshly isolated hepatocyte suspensions demonstrated high flux control coefficients for β -oxidation of 0.5 mM palmitate in cells from fed, fasted, re-fed and insulin-treated fed rats [18].

Our aim was to determine the flux control coefficient of CPT I for palmitate oxidation in hepatocyte cultures at low and high palmitate concentrations in order to simulate substrate concen-

trations in fed and fasted states. We preincubated hepatocyte cultures with 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate (*RS*-Etomoxir) which is metabolised to *RS*-Etomoxir-CoA, the *R*-enantiomer of which is an irreversible inhibitor of CPT I [19], and measured enzyme activity and flux through β -oxidation. The flux control coefficient was calculated from the fractional inhibition of flux and enzyme activity. The results showed that the flux control coefficient is insensitive to palmitate concentration. This contrasts with the shift in control in isolated mitochondria [15].

MATERIALS AND METHODS

Materials

RS(rac)-Etomoxir (sodium salt) was generously provided by Dr. H. P. O. Wolf (Byk Gulden Chemische Fabrik GmbH, Konstanz, Germany). Collagenase (type IV) and biochemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.). Defatted BSA was from Advanced Protein Products (Brierley Hill, West Midlands, U.K.). Radiochemicals were from Amersham International (Amersham, Little Chalfont, Bucks., U.K.). [3 H]Carnitine was washed with water-saturated butanol before use to remove impurities.

Hepatocyte cultures

Hepatocytes were isolated from fed male Wistar rats (250–300 g) by collagenase perfusion and cultured in monolayer in serum-free medium for 16–24 h to allow recovery of normal metabolism [20]. Cell protein was determined by an automated Lowry method [21].

Incubation with Etomoxir for determination of β -oxidation or CPT I activity

Hepatocyte monolayers were incubated in fresh medium containing various concentrations of *RS*-Etomoxir, for 30 min. This medium was then removed and the cells were washed with

Abbreviations used: CPT, carnitine palmitoyltransferase; Etomoxir, 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate; $I_{\max}$ for an irreversible inhibitor, the minimum concentration of inhibitor causing total inhibition of enzyme; IC_{50} , concentration of inhibitor giving half maximal inhibition; LCFA, long-chain fatty acid; MCA, Metabolic Control Analysis.

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Figure 1 Sensitivity of CPT I to inhibition by Etomoxir in rat hepatocyte monolayers

Hepatocytes were preincubated for 30 min with various concentrations of Etomoxir before permeabilization of the cells and assay of CPT I activity. Values are the means $\pm$ S.E.M. for five different hepatocyte preparations.

150 mM NaCl. The monolayers were incubated for 1 h in fresh medium (without inhibitor), which contained either 0.2 mM or 1 mM [U - ^{14}C]palmitate (0.15 μ Ci/ml), 2% (w/v) defatted BSA and 0.5 mM carnitine for determination of β -oxidation or with the same additions but without radiolabel for subsequent determination of CPT I activity.

Measurement of [U - ^{14}C]palmitate oxidation

For determination of β -oxidation, the medium was collected at the end of the 1 h incubation period, deproteinized with $HClO_4$, and the radioactivity incorporated into acid-soluble metabolites was determined [22]. $^{14}CO_2$ production was only about 5% of the labelled products and was disregarded [22]. Control rates of β -oxidation were linear for at least 90 min (results not shown), and are expressed as nmol of palmitate metabolized/min per mg of cellular protein.

Measurement of CPT I activity

CPT I activity was determined in digitonin-permeabilized hepatocytes as described in [23], either directly on termination of the incubations with Etomoxir (Figure 1), or after incubation (1 h) in medium containing palmitate in experiments parallel to those for β -oxidation (see Figure 3).

Calculation of the flux control coefficient of CPT I

This was determined either from the profile of inhibition of palmitate β -oxidation by *RS*-Etomoxir (method 1), or from the fractional inhibition of β -oxidation relative to the fractional inhibition of CPT I (method 2).

Method 1: for an irreversible inhibitor, Groen et al. [24] have shown that an estimate of the flux control coefficient, C_E^J , of the inhibited enzyme is given by:

$$C_E^J = -\frac{\delta J/J}{\delta I/I_{\max}} \quad (1)$$

where J is the flux, I the inhibitor concentration and $I_{\max}$ the minimum inhibitor concentration that will totally inhibit the

enzyme. The flux control coefficient can then be measured from a plot of β -oxidation of palmitate versus [*RS*-Etomoxir].

Method 2: the flux control coefficient as defined by Mazat et al. [25] can also be determined from the effects of an inhibitor both on pathway flux and on the enzyme activity, when [inhibitor] tends to zero:

$$C_E^J = \frac{\delta \ln J / \delta I}{\delta \ln V / \delta I} \quad (2)$$

and represents the ratio of the initial slope of the inhibition curve of the flux (J), under steady-state conditions, to the initial slope of the inhibition curve of the isolated enzyme step (V). The results are expressed as a ratio of the initial slope of the inhibition curve of β -oxidation to the initial slope of the inhibition curve of CPT I.

RESULTS AND DISCUSSION

Sensitivity of CPT activity to Etomoxir

To test the validity of the CPT assay, the inhibition by malonyl-CoA (added after permeabilization) was compared with the inhibition after preincubation with Etomoxir. Malonyl-CoA (100 μ M) inhibited CPT activity by 78% (1.13 ± 0.05 to 0.25 ± 0.03 nmol/min per mg of protein). The remaining CPT activity may be due to mitochondrial CPT II and malonyl-CoA-insensitive carnitine acyltransferases in peroxisomes [26] or endoplasmic reticulum [27]. Figure 1 shows the inhibition of CPT by *RS*-Etomoxir. Up to 75% of the total CPT activity was sensitive to inhibition by 2 μ M *RS*-Etomoxir. The concentration of *RS*-Etomoxir that gave 50% inhibition of CPT I was 50 nM. The sensitivity of CPT activity to Etomoxir suggests it is predominantly mitochondrial CPT I that is inhibited by Etomoxir, since the mitochondrial enzyme has a much higher affinity for Etomoxir-CoA ($IC_{50} < 10$ nM, [28]) than the micro-

Figure 2 Effect of *RS*-Etomoxir on the rate of palmitate oxidation in rat hepatocyte monolayers: calculation of flux control coefficient using method 1

Rat hepatocyte monolayers were preincubated with various concentrations of *RS*-Etomoxir (30 min) before incubation with culture medium containing 0.5 mM L-carnitine, 2% (w/v) BSA and either 0.2 or 1 mM [U - ^{14}C]palmitate. Values are the means $\pm$ S.E.M. for $n = 3$ –5 different hepatocyte preparations. Estimates of $I_{\max}$ are 70 and 85 nM for 0.2 mM ($\circ$) and 1 mM palmitate ($\bullet$) respectively. The initial slope ($\delta J/\delta I$) of the graph was -0.0017 and -0.0066 nmol (min $\cdot$ mg) $^{-1} \cdot$ (nM) $^{-1}$ for 0.2 mM and 1 mM palmitate. The insert is an expansion of the graph at low Etomoxir concentrations.

Figure 3 Inhibition of CPT I activity and β -oxidation by Etomoxir: calculation of flux control coefficient using method 2

Hepatocyte monolayers were incubated with Etomoxir for 30 min, and CPT I activity ($\square$) and β -oxidation ($\blacktriangle$) from (upper panel) 0.2 mM palmitate or (lower panel) 1 mM palmitate were measured as described in the Materials and methods section. The initial slope of each inhibition curve was fitted by linear regression between 0 and 20 nM Etomoxir (best linear fit extrapolation to zero). Each point represents the percentage inhibition by Etomoxir of either CPT activity or β -oxidation; the error bars have been omitted for clarity (S.E.M. was less than 8%). The uninhibited rates of β -oxidation were 0.18 ± 0.03 (upper panel) and 0.81 ± 0.11 (lower panel) nmol of palmitate/min per mg of protein (mean $\pm$ S.E.M., $n = 3-4$). The uninhibited CPT activity under similar conditions was 0.67 ± 0.02 and 0.80 ± 0.11 nmol/min per mg of protein (mean $\pm$ S.E.M., $n = 3$) respectively.

somal enzyme ($IC_{50} \sim 700$ nM, [29]), and the peroxisomal enzyme is not irreversibly inhibited by this compound [28]. In this study we assume that Etomoxir-sensitive CPT activity and β -oxidation are due only to the activity of mitochondrial CPT I.

Flux control coefficients calculated by Etomoxir inhibition of flux through palmitate β -oxidation (method 1)

We used MCA to measure the regulatory potential of CPT I on long-chain-fatty-acid (LCFA) β -oxidation in hepatocyte monolayers at 0.2 mM and 1.0 mM palmitate. Rates of β -oxidation were 4.5 times higher at 1.0 mM palmitate than at 0.2 mM palmitate. Figure 2 shows the inhibition of palmitate β -oxidation with increasing concentrations of *RS*-Etomoxir. At each palmitate concentration, about 20 % of palmitate β -oxidation was not inhibited by Etomoxir. This Etomoxir-insensitive β -oxidation may be due to carnitine-independent uptake of LCFAs into

mitochondria and conversion into CoA esters by the medium-chain CoA synthase.

To calculate the flux control coefficient, values for the $I_{\max}$ were obtained by extrapolation of the flux against the inhibitor curve (Figure 2) to the point of residual β -oxidation. $I_{\max}$ values were 70 nM and 85 nM for 0.2 mM and 1.0 mM palmitate respectively. The initial slope of the inhibition curve was calculated by linear regression of the first five points. Substitution of the values for $\delta J/\delta I$, J and $I_{\max}$ into eqn. (1) gave similar control coefficients of 0.67 and 0.68 at 0.2 mM and 1.0 mM palmitate respectively, despite the different values of $I_{\max}$ and $\delta J/\delta I$ (see Figure 2). The estimate of the flux control coefficient by this method is approximate [30], and is also sensitive to errors in $I_{\max}$.

Flux control coefficients calculated from the fractional effect of Etomoxir on CPT I activity and on flux through β -oxidation (method 2)

To obviate the need to measure $I_{\max}$, the flux control coefficient for CPT I was estimated also from the fractional effect of Etomoxir both on CPT I activity and on flux through palmitate β -oxidation. Figure 3 shows the inhibition by *RS*-Etomoxir of CPT I activity and β -oxidation. The flux control coefficient was determined from the initial slopes of the inhibition curves which had been extrapolated back to zero Etomoxir concentration. This gave flux control coefficients for CPT I of 0.79 and 0.77 for β -oxidation at 0.2 mM and 1 mM palmitate respectively.

Conclusions

Although there are large differences in flux through β -oxidation at 1 mM when compared with 0.2 mM palmitate, the control coefficients of CPT I are very similar. Flux control coefficients may vary with physiological conditions through changes in enzyme activities and metabolite concentrations and also with the conditions *in vitro* (e.g. freshly isolated cells versus monolayer cultures) used for the experimental study. Drynan and colleagues [18] reported similar flux control coefficients to those of the present study for CPT I activity in fresh hepatocyte suspensions isolated from fed, fasted or fasted-refed rats which had been incubated with 0.5 mM palmitate. These results, taken together, indicate that the flux control coefficient of CPT I is similar in freshly isolated and cultured hepatocytes, it is independent of the nutritional state of the donor rat [18], and it is insensitive to changes in fatty acid concentration. It can be concluded, therefore, that CPT I plays an important role in controlling flux through LCFA oxidation and that control is not transferred to other β -oxidation enzymes with changing substrate concentration or nutritional state.

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