Probing peroxisomal β -oxidation and the labelling of acetyl-CoA proxies with [1- ^{13}C]octanoate and [3- ^{13}C]octanoate in the perfused rat liver

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We reported previously that a substantial fraction of the acetyl groups used to synthesize malonyl-CoA in rat heart is derived from peroxisomal β -oxidation of long-chain and very-long-chain fatty acids. This conclusion was based on the interpretation of the ^{13}C -labelling ratio (malonyl-CoA)/(acetyl moiety of citrate) measured in the presence of substrates that label acetyl-CoA in mitochondria only (ratio < 1.0) or in both mitochondria and peroxisomes (ratio > 1.0). The goals of the present study were to test, in rat livers perfused with [1- ^{13}C]octanoate or [3- ^{13}C]octanoate, (i) whether peroxisomal β -oxidation contributes acetyl groups for malonyl-CoA synthesis, and (ii) the degree of labelling homogeneity of acetyl-CoA proxies (acetyl moiety of citrate, acetate, β -hydroxy-

butyrate, malonyl-CoA and acetylcarnitine). Our data show that (i) octanoate undergoes two cycles of peroxisomal β -oxidation in liver, (ii) acetyl groups formed in peroxisomes contribute to malonyl-CoA synthesis, (iii) the labelling of acetyl-CoA proxies is markedly heterogeneous, and (iv) the labelling of C1 + 2 of β -hydroxybutyrate does not reflect the labelling of acetyl-CoA used in the citric acid cycle.

Key words: acetylcarnitine, acetyl-CoA, fatty acid oxidation, β -hydroxybutyrate, malonyl-CoA, metabolic channelling, peroxisome.

INTRODUCTION

Peroxisomal β -oxidation is classically described as a process by which very-long-chain n -fatty acids are partially shortened to long-chain acyl-CoAs which are transferred to the mitochondria for complete β -oxidation [1]. Based on the relative activities of enzymes of mitochondrial compared with peroxisomal β -oxidation, the latter appears to contribute only a small fraction of total fatty acid oxidation in liver. The peroxisome is the only known site of β -oxidation of n -dicarboxylates. There is evidence that octanoate, a medium-chain fatty acid, is oxidized to some extent in peroxisomes. Skorin et al. [2] showed that the production of H_2O_2 by hepatocytes, incubated in the presence of inhibitors of carnitine palmitoyltransferase I, was increased by octanoate. We reported previously that, in rat livers perfused with non-recirculating buffer containing [1- ^{13}C]octanoate, the enrichment of acetate released in the perfusate was 35 % [3]. Since the complete β -oxidation of 1 mol of octanoate yields 4 mol of acetyl-CoA, the maximal enrichment of mitochondrial acetyl-CoA derived from [1- ^{13}C]octanoate is 25 %. Since 35 % enriched acetate could not be derived from the hydrolysis of mitochondrial acetyl-CoA, we concluded that (i) acetate was derived from the hydrolysis of highly labelled peroxisomal acetyl-CoA, and (ii) octanoate undergoes at least one cycle of peroxisomal β -oxidation in rat liver.

We showed recently that, in the heart, acetyl-CoA derived from the partial peroxisomal β -oxidation of long-chain and very-long-chain fatty acids is used for malonyl-CoA synthesis [4], a key regulator of the mitochondrial oxidation of long-chain fatty acids [5,6]. This conclusion was based on the comparison of the ^{13}C -labelling of malonyl-CoA and the acetyl moiety of citrate, a proxy for mitochondrial acetyl-CoA [4]. The labelling ratio (malonyl-CoA)/(acetyl moiety of citrate) was < 1.0 in the presence of substrates which yield labelled acetyl-CoA in mitochondria

only ([16- ^{13}C]palmitate, [U- $^{13}\text{C}_6$]glucose and [3- ^{13}C]lactate + [3- ^{13}C]pyruvate). In contrast, the labelling ratio was > 1.0 in the presence of fatty acids which yield labelled acetyl-CoA in both mitochondria and peroxisomes ([1,2- $^{13}\text{C}_2$]palmitate, [1- ^{13}C]oleate and [1,2,3,4- $^{13}\text{C}_4$]docosanoate). This demonstrated that a fraction of the acetyl-CoA used for malonyl-CoA synthesis is formed in the extramitochondrial space, most likely in peroxisomes.

In the present study, we used the (malonyl-CoA)/(acetyl moiety of citrate) labelling ratio to investigate the peroxisomal oxidation of octanoate in perfused rat livers. We perfused rat livers with various concentrations of [1- ^{13}C]octanoate or [3- ^{13}C]octanoate and measured the ^{13}C -labelling of malonyl-CoA, the acetyl moiety of citrate, free acetate, acetylcarnitine and the C1 + 2 fragment of ketone bodies (another proxy for the labelling of mitochondrial acetyl-CoA in liver [7,8]). This protocol allowed us to test the degree of labelling homogeneity of the various acetyl-CoA proxies.

MATERIALS AND METHODS

Materials

Chemicals and biochemicals were obtained from Sigma–Aldrich. [1- ^{13}C]Octanoate, [1- ^{13}C]hexyl bromide, [U- $^{13}\text{C}_3$]malonic acid, [1- ^{13}C]acetate and [$^2\text{H}_6$, 1,1'- $^{13}\text{C}_2$]acetic anhydride were purchased from Isotec (Miamisburg, OH, U.S.A.). [3- ^{13}C]Octanoate was prepared from [1- ^{13}C]hexyl bromide by malonic acid synthesis [9]. Mass spectrometric analysis of [3- ^{13}C]octanoate confirmed the complete decarboxylation of [1- ^{13}C]hexylmalonate, an intermediate of the synthesis. [1- ^{13}C]Acetyl-CoA was prepared from [1- ^{13}C]acetate as in [10]. [$^2\text{H}_3$, 1- ^{13}C]Acetyl-CoA was prepared by reacting [$^2\text{H}_6$, 1,1'- $^{13}\text{C}_2$]acetic anhydride with CoA [11]. The two labelled acetyl-CoAs were purified by HPLC [12].

Abbreviations used: BHB, β -hydroxybutyrate; GC-MS, gas chromatography–MS; LC-MS, liquid chromatography–MS; MPE, molar percentage ^{13}C -enrichment.

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Liver perfusion experiments

Livers from overnight-fasted Sprague–Dawley male rats (170–220 g) were perfused as outlined in [13] with non-recirculating bicarbonate buffer containing 4 mM glucose. After 15 min of equilibration, 0–1.0 mM of [1-¹³C]octanoate or [3-¹³C]octanoate was added to the perfusate. The livers were quick-frozen 30 min after the addition of the labelled substrate.

Analytical procedures

We developed a new assay for the concentration and ¹³C-labelling pattern of free acetyl-CoA. Powdered frozen liver (150–200 mg) was spiked with 10 nmol of [²H₃,1-¹³C]acetyl-CoA tissue and extracted for 5 min with 1 ml of 6% HClO₄, using a Polytron homogenizer. After centrifugation, the extract was slowly pushed through an Oasis cartridge (Waters) pre-washed with 1 ml of methanol and 1 ml of water. This cartridge traps low-molecular-mass water-soluble CoA esters. After rinsing the cartridge with 1 ml of 5% methanol in water, acetyl-CoA was eluted with 1 ml of methanol. After solvent evaporation at 5 °C, the residue was dissolved in 0.5 ml of 100 mM potassium phosphate buffer, pH 8.5, and reacted with 0.15 ml of 1 mM thiophenol solution in tetrahydrofuran (dried on sodium, freshly distilled and tested for the absence of acetylthiophenol) at 60 °C for 4 h. Excess thiophenol was precipitated with 0.1 ml of 5 mM silver nitrate. Acetylthiophenol was extracted with three 4 ml volumes of diethyl ether. The extract was dried with Na₂SO₄, and the solvent was evaporated down to approx. 0.1 ml to avoid evaporation of volatile acetylthiophenol. The acetylthiophenol solution in ether was analysed by ammonia-positive chemical ionization GC-MS (gas chromatography–MS). Ions at *m/z* 170 (*M* + NH₄⁺) to 174 (*M* + NH₄⁺ + 4) were monitored.

The ¹³C-labelling of the acetyl moiety of liver citrate (a probe of mitochondrial acetyl-CoA) was assayed as outlined in [4] by cleaving citrate with ATP citrate-lyase isolated from rat liver [14] and reacting the acetyl-CoA formed with thiophenol, as above. The ¹³C-labelling of the C1 + 2 fragment of BHB (another probe of mitochondrial acetyl-CoA [7,8]) was calculated as the difference between the labelling of the whole molecule and that of the C3 + 4 fragment [15]. The ¹³C-labelling of liver malonyl-CoA [12] and of acetate in the effluent perfusate [16] were assayed as described previously. The labelling of acetylcarnitine was measured by LC-MS (liquid chromatography–MS) [17].

Data presentation and statistics

For each protocol, we ran seven perfusions in the presence of increasing concentrations of [1-¹³C]octanoate or [3-¹³C]octanoate. Data shown in Figures are the MPE (molar percentage ¹³C-enrichments) of the acetyl-CoA proxies measured in a given liver perfusion. MPE is defined as the mol fraction percentage of analyte containing one ¹³C atom, above natural enrichment [18]. They represent means of duplicate GC-MS or LC-MS injections respectively, which differed by < 2%. Each symbol in the Figures corresponds to one perfusion experiment. The statistical significance of differences between the profiles of ¹³C-enrichments of the acetyl-CoA proxies in each set of experiments was tested using Student's paired *t* test (Graph Pad Prism Software, Version 3.0).

RESULTS AND DISCUSSION

We had described previously an assay of the ¹³C-enrichment of acetyl-CoA after conversion into acetylglycine or acetylsarcosine [19]. Acetylsarcosine was more practical than acetylglycine, because it can be extracted in organic solvent. However, we found

Figure 1 Profiles of the MPE of acetyl-CoA or its proxies as a function of the concentration of [1-¹³C]octanoate in the perfusate

The enrichment of total acetyl-CoA, malonyl-CoA, the acetyl moiety of citrate and acetylcarnitine were assayed in the frozen liver tissue. The enrichment of free acetate and of the C1 + 2 fragment of BHB was assayed in the effluent perfusate. In this and subsequent Figures, each symbol refers to one perfused liver.

that the lots of commercial sarcosine that we tested contained a small amount of acetylsarcosine. This resulted in an artifactual decrease in the measured enrichment of tissue acetyl-CoA. This is why we developed an assay of acetyl-CoA enrichment by transesterification with thiophenol. With this assay, calibration curves of the MPE of acetyl-CoA, assayed by GC-MS after transesterification with thiophenol, are linear ($r^2 = 0.99$; results not shown). The concentration of acetyl-CoA can also be assayed using an internal standard of [²H₃,1-¹³C]acetyl-CoA (results not shown). To check whether thiophenol can react with free acetate, we incubated 10 nmol of unlabelled acetyl-CoA and 100 nmol of [1,2-¹³C₂]acetate with thiophenol. We did not detect any M2 enrichment of acetylthiophenol above the M2 natural enrichment. Thus, under our conditions, the assay of acetyl-CoA enrichment is not affected by free acetate, an ubiquitous contaminant. We described previously an assay of the enrichment of acetyl-CoA by LC-MS of the whole molecule [4]. The acetylthiophenol technique allows assaying the enrichment of acetyl-CoA by GC-MS which is more generally available than LC-MS. Also the basal enrichment of acetylthiophenol is 10.3% compared with that of intact acetyl-CoA (26.6%). However, the LC-MS technique is more sensitive than the GC-MS technique.

In order to test whether octanoate undergoes one or two cycles of peroxisomal β -oxidation, we perfused rat livers with increasing concentrations of [1-¹³C]octanoate or [3-¹³C]octanoate. We assayed the MPE of a number of compounds that are closely related to acetyl-CoA, i.e. malonyl-CoA, the acetyl moiety of citrate, free acetate, acetylcarnitine and the C1 + 2 fragment of BHB (Figures 1 and 2).

Starting with proxies of the labelling of mitochondrial acetyl-CoA, the labelling profile of the C1 + 2 fragment of BHB was significantly higher than that of the acetyl moiety of citrate, in the presence of [1-¹³C]octanoate ($P = 0.011$) (Figure 1) or

Figure 2 Profiles of the MPE of acetyl-CoA or its proxies as a function of the concentration of $[3-^{13}\text{C}]$ octanoate in the perfusate

See legend to Figure 1 for details.

Figure 3 Profiles of the labelling ratio (C1 + 2 of BHB)/(acetyl moiety of citrate) as a function of the concentration of $[^{13}\text{C}]$ octanoate in the perfusate

$[3-^{13}\text{C}]$ octanoate ($P = 0.027$) (Figure 2). Figure 3 shows that the labelling ratio (C1 + 2 of BHB)/(acetyl moiety of citrate) decreases progressively towards 1.0 as the fatty acid concentration increases.

Hetenyi et al. [7] and Katz [8] have proposed that the labelling of the C1 + 2 fragment of BHB could be used as a proxy for the labelling of liver mitochondrial acetyl-CoA. In the present study, and a previous study [20], we were able to compare the ^{13}C -labelling of the C1 + 2 fragment of BHB and of the acetyl moiety of citrate. We showed previously that, in livers perfused with 0.2 mM $[1-^{13}\text{C}]$ octanoate, the MPEs of the acetyl moiety of citrate and C1 + 2 of BHB were slightly, but significantly, different (19.9 compared with 23%; $P < 0.01$) [20]. The present study expands on this finding by testing the influence of $[^{13}\text{C}]$ octanoate

concentration on the labelling of the two acetyl-CoA proxies. At very low concentrations of $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate, the labelling of the C1 + 2 fragment of BHB was < 3.5 times greater than that of the acetyl moiety of citrate (Figures 1–3). When the concentration of the $[1-^{13}\text{C}]$ octanoate increased, the labelling ratio (C1 + 2 fragment of BHB)/(acetyl moiety of citrate) decreased towards 1.0.

The discrepancy between the labelling of the C1 + 2 fragment of BHB and that of the acetyl moiety of citrate probably results from associations of enzymes resulting in metabolic channelling of intermediates [21,22], especially at low flux rate. Previous studies had concluded that the labelling of liver mitochondrial acetyl-CoA is not homogeneous [23,24]. However, to our knowledge of the literature, this is the first demonstration of the influence of the concentration of a labelled acetyl-CoA precursor on the labelling homogeneity of mitochondrial acetyl-CoA. Our data thus show that the labelling of the C1 + 2 fragment of BHB is not a suitable proxy for that of mitochondrial acetyl-CoA when labelled acetyl-CoA is derived from a low concentration of a fatty acid labelled in its first or second acetyl moiety.

The labelling of total liver acetyl-CoA was greater than that of the acetyl moiety of citrate ($P = 0.02$) (Figures 1 and 2). Thus some pool(s) of extramitochondrial acetyl-CoA is (are) more labelled than the mitochondrial acetyl-CoA that forms citrate. Soboll et al. [25] have shown that more than 90 % of liver acetyl-CoA is mitochondrial. Thus, under our conditions, the small fraction of liver acetyl-CoA that is extramitochondrial must be strongly labelled. This extramitochondrial acetyl-CoA cannot be entirely derived from mitochondrial acetyl-CoA transferred to the cytosol via citrate and ATP citrate-lyase [26]. This confirms that part of the extramitochondrial acetyl-CoA is derived from the peroxisomal β -oxidation of $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate.

Livers perfused with $[1-^{13}\text{C}]$ octanoate released acetate, the enrichment of which reached a higher plateau than that of total acetyl-CoA and of all the acetyl-CoA proxies measured (Figure 1). The profiles of acetate labelling were significantly higher than those of malonyl-CoA and the acetyl moiety of citrate ($P < 0.05$). The concentration of acetate was < 0.05 mM in the effluent perfusate. Free $[^{13}\text{C}]$ acetate presumably results from the hydrolysis of $[1-^{13}\text{C}]$ acetyl-CoA by a peroxisomal acyl-CoA hydrolase [1,27]. Leighton et al. [28] demonstrated the production of $[^{14}\text{C}]$ acetate by hepatocytes incubated with $[1-^{14}\text{C}]$ dodecanedioate, a substrate which is oxidized exclusively in peroxisomes. The production of $[1-^{13}\text{C}]$ acetate from $[1-^{13}\text{C}]$ octanoate cannot be explained by the hydrolysis of mitochondrial acetyl-CoA, the enrichment of which reaches a plateau at 16–18 % (Figure 1), while the enrichment of acetate reaches a plateau at 33 %. Also, 33 % enriched acetate cannot be derived from the oxidation of acetone [29,30] (formed by acetoacetate decarboxylation), since the enrichment of acetone could not be greater than that of the C1 + 2 moiety of BHB (Figure 1).

We considered the possibility that, in the presence of $[1-^{13}\text{C}]$ octanoate, malonyl-CoA could be labelled via $[1-^{13}\text{C}]$ acetate (rather than $[1-^{13}\text{C}]$ acetyl-CoA) released from peroxisomes after activation by cytosolic acetyl-CoA synthetase. To test this possibility, we perfused livers with 1 mM unlabelled octanoate and 0.05 mM of 99 % enriched $[1-^{13}\text{C}]$ acetate. The concentration of $[1-^{13}\text{C}]$ acetate was chosen as the maximal concentration released by livers perfused with $[1-^{13}\text{C}]$ octanoate. The enrichment of infused $[1-^{13}\text{C}]$ acetate was triple that of the maximal enrichment of acetate released by the liver in the presence of 1 mM $[1-^{13}\text{C}]$ octanoate (Figure 1). We could not detect any labelling in malonyl-CoA, acetyl moiety of citrate or BHB, presumably because of the low concentration of $[1-^{13}\text{C}]$ acetate used. Therefore we can exclude that, in the presence of $[1-^{13}\text{C}]$ octanoate, malonyl-CoA becomes

Figure 4 Profiles of the labelling ratio (malonyl-CoA)/(acetyl moiety of citrate) as a function of the concentration of $[^{13}\text{C}]$ octanoate in the perfusate

labelled via activation of free $[1-^{13}\text{C}]$ acetate derived from peroxisomal acetyl-CoA.

To test whether octanoate undergoes more than one cycle of peroxisomal β -oxidation, we perfused livers with $[3-^{13}\text{C}]$ octanoate, which forms $[1-^{13}\text{C}]$ hexanoyl-CoA in peroxisomes, a possible precursor of peroxisomal $[1-^{13}\text{C}]$ acetyl-CoA. Livers perfused with $[3-^{13}\text{C}]$ octanoate also released acetate, the enrichment of which reached a higher plateau than that of total acetyl-CoA and of all the acetyl-CoA proxies measured (Figure 2). This suggests strongly that $[3-^{13}\text{C}]$ octanoate can undergo two cycles of peroxisomal β -oxidation in rat liver.

In the presence of $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate, the labelling ratios (malonyl-CoA)/(acetyl moiety of citrate) were not significantly different from 1.0, but were significantly from each other ($P < 0.01$) (Figure 4). This observation alone would not reflect peroxisomal β -oxidation of $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate. However, the high enrichment of acetate released by livers perfused with $[1-^{13}\text{C}]$ octanoate (Figure 1) or $[3-^{13}\text{C}]$ octanoate (Figure 2) shows that some acetyl-CoA is formed in peroxisomes from the first and from the second acetyl moiety of octanoate. The enrichment of free acetate is greater in the presence of $[1-^{13}\text{C}]$ octanoate compared with $[3-^{13}\text{C}]$ octanoate. Thus peroxisomal $[^{13}\text{C}]$ acetyl-CoA derived from $[3-^{13}\text{C}]$ octanoate is presumably more diluted than that derived from $[1-^{13}\text{C}]$ octanoate before it is hydrolysed. This dilution results from the formation of unlabelled acetyl-CoA from the peroxisomal β -oxidation of unlabelled endogenous long-chain fatty acids. It thus appears that, although octanoate can undergo two cycles of peroxisomal β -oxidation in liver (compared with one cycle in heart [31]), a greater fraction of the first acetyl of octanoate undergoes peroxisomal β -oxidation compared with the second acetyl.

We assayed the enrichment of acetylcarnitine, in the hope that it would shed some light on the mechanism by which acetyl groups derived from peroxisomal β -oxidation leave the peroxisomes. Figure 5(A) shows that, in livers perfused with increasing concentrations of $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate, the labelling ratios (acetylcarnitine)/(total acetyl-CoA) are very close to 1.0 and are not significantly different. Thus the labelling of liver acetylcarnitine reflects that of total tissue acetyl-CoA, with the mitochondrial and extramitochondrial pools mixed during the extrac-

Figure 5 Profiles of the labelling ratio acetylcarnitine/(total acetyl-CoA) (A) and acetylcarnitine/(acetyl of citrate) (B) as a function of the concentration of $[^{13}\text{C}]$ octanoate in the perfusate

Figure 6 Profiles of the labelling ratio (malonyl-CoA)/(C1 + 2 of BHB) as a function of the concentration of $[^{13}\text{C}]$ octanoate in the perfusate

tion procedure. In contrast, the labelling ratio (acetylcarnitine)/(acetyl moiety of citrate) appears to stabilize at approx. 1.7 with $[1-^{13}\text{C}]$ octanoate, but at approx. 1.3 with $[3-^{13}\text{C}]$ octanoate (Figure 5B). If acetyl groups derived from peroxisomal β -oxidation of $[1-^{13}\text{C}]$ octanoate left the peroxisomes as acetylcarnitine, one would indeed expect that the total tissue acetylcarnitine would be more labelled than the acetyl moiety of citrate. The same rationale applies to $[^{13}\text{C}]$ acetyl-CoA formed in peroxisomes from $[3-^{13}\text{C}]$ octanoate.

Figure 6 shows that, in livers perfused with $[1-^{13}\text{C}]$ octanoate or $[3-^{13}\text{C}]$ octanoate, the labelling ratios (malonyl-CoA)/(C1 + 2 of BHB) are < 1.0 at substrate concentrations lower than 1 mM, and are not significantly different. This confirms that the labelling of

the C1 + 2 of BHB does not reflect, in most cases, that of acetyl groups exported from mitochondria to cytosol.

In conclusion, the present study demonstrates the labelling heterogeneity of acetyl-CoA pools and their proxies in rat liver. This heterogeneity is most pronounced at low concentration of the labelled precursor. The labelling of the C1 + 2 moiety of BHB does not reflect that of the acetyl-CoA that enters the citric acid cycle. Our data also clearly demonstrate that octanoate can undergo two cycles of peroxisomal β -oxidation in liver, compared with one cycle in heart [31]. The relative labelling of the acetyl moiety of citrate and free acetate allows us to probe peroxisomal fatty acid oxidation in liver.

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