Regulatory effects of fatty acids on decarboxylation of leucine and 4-methyl-2-oxopentanoate in the perfused rat heart

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The regulatory effects of fatty acids on the oxidative decarboxylation of leucine and 4-methyl-2-oxopentanoate were investigated in the isolated rat heart. Infusion of the long-chain fatty acid palmitate resulted in both an inactivation of the branched-chain 2-oxo acid dehydrogenase and an inhibition of the measured metabolic flux through this enzyme complex. Pyruvate addition also caused both an inactivation and an inhibition of the flux through the complex. On the other hand, the medium-chain fatty acid octanoate caused an activation of and a stimulation of flux through the branched-chain 2-oxo acid dehydrogenase when the perfusion conditions before octanoate addition maintained the enzyme complex in its inactive state. When the enzyme complex was activated before octanoate infusion, this fatty acid caused a significant inhibition of the flux through the branched-chain 2-oxo acid dehydrogenase reaction. Inclusion of glucose in the perfusion medium prevented the octanoate-mediated activation of the branched-chain 2-oxo acid dehydrogenase.

Branched-chain amino acids are readily metabolized in extra-hepatic tissues, and several experimental observations have led to the conclusion that muscle is the primary site of branched-chain amino acid transamination (Miller, 1961; Manchester, 1965; Buse *et al.*, 1972; Odessey & Goldberg, 1972; Goldberg & Chang, 1978). Transamination by the specific cytosolic aminotransferase (EC 2.6.1.42), with 2-oxoglutarate as the amino-group acceptor, is followed by oxidative decarboxylation of the resulting branched-chain 2-oxo acid by the mitochondrial branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.4), generating the corresponding branched-chain 2-oxo acyl-CoA derivative, NADH and CO₂. In muscle tissue the branched-chain 2-oxo acid dehydrogenase complex is the rate-limiting enzyme in the breakdown of branched-chain amino acids (Goldberg & Chang, 1978; Odessey & Goldberg, 1979; Hutson *et al.*, 1978, 1980), in contrast with liver, where transamination is rate-limiting (Shinnick & Harper, 1976).

The branched-chain 2-oxo acid dehydrogenase is regulated in an analogous manner to the structurally similar pyruvate dehydrogenase. The enzyme is subject to feedback inhibition by its products, branched-chain acyl-CoA and NADH (Pettit *et al.*, 1978; Parker & Randle, 1978*a,b*). In addition,

regulation of the enzyme by phosphorylation/dephosphorylation has been demonstrated in mitochondria (Lau *et al.*, 1981; Buffington *et al.*, 1981; Randle *et al.*, 1981; Patel & Olson, 1982), in the purified enzyme (Fatania *et al.*, 1981; Odessey, 1982; Harris *et al.*, 1982; Paxton & Harris, 1982), and in the perfused rat heart (Buxton & Olson, 1982).

Studies in several laboratories have provided apparently conflicting results concerning the effects of fatty acids on the metabolism of branched-chain amino acid in muscle tissue. Buse *et al.* (1972) demonstrated a stimulation of branched-chain amino acid oxidation by octanoate and palmitate in the rat hemidiaphragm and by octanoate in the perfused rat heart. Others have observed stimulation of branched-chain amino acid metabolism by butyrate or octanoate in skeletal-muscle preparations, but no stimulation, or even inhibition, by longer-chain-length fatty acids (Paul & Adibi, 1976; Spydevold & Hokland, 1981). In the perfused heart, Sans *et al.* (1980) showed a marked inhibition of leucine decarboxylation by palmitate, and branched-chain 2-oxo acid oxidation was inhibited dramatically by octanoate (Buffington *et al.*, 1979; Waymack *et al.*, 1980).

The present studies in the perfused heart were initiated in an attempt to resolve these apparently contradictory findings.

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Experimental

Male Sprague-Dawley rats (200–300g), fed *ad libitum*, were anaesthetized by intraperitoneal injection of sodium pentobarbital (10mg/100g body wt.). The hearts were excised rapidly and placed into Krebs-Henseleit (1932) bicarbonate buffer with the bicarbonate concentration decreased to one-third of normal to give pH 7.6 at 4°C without gassing. The hearts were trimmed to remove fat and connective tissue, and then non-recirculating Langendorff (1895) perfusion was established, with Krebs-Henseleit bicarbonate buffer with the Ca^{2+} concentration lowered to 1.25 mM. Perfusions were performed at 37°C at a flow rate of 10 ml/min. Hearts were wash-out perfused for 5 min before the collection of samples and the infusion of ^{14}C -labelled substrate were initiated; unlabelled substrate was present during the wash-out. Effluent perfusate was collected at 30 s intervals, and $^{14}\text{CO}_2$ produced by the heart from ^{14}C -labelled substrate was determined as described previously (Buffington *et al.*, 1979). ^{14}C -labelled 2-oxo acids, produced by the heart from infused ^{14}C -labelled amino acids, were determined by the method of Odessey & Goldberg (1979). Leucine decarboxylation is defined as the rate of production of $^{14}\text{CO}_2$ from infused ^{14}C -leucine via transamination and subsequent oxidative decarboxylation by the branched-chain 2-oxo acid dehydrogenase.

Measurement of branched-chain 2-oxo acid dehydrogenase activity in freeze-clamped hearts was performed as described by Waymack *et al.* (1980), except that 3-methyl-2-oxo[^{14}C]butyrate was used as the substrate in place of 4-methyl-2-oxo[^{14}C]pentanoate.

Palmitate was bound to defatted 3% (w/v) bovine serum albumin by the method of Evans *et al.* (1963) before infusion.

[^{14}C]Leucine, [^{14}C]valine and Aquasol were obtained from New England Nuclear. Phenethylamine was purchased from Research Products International and Fisher Scientific. 4-Methyl-2-oxo[^{14}C]pentanoate and 3-methyl-2-oxo[^{14}C]butyrate were prepared from [^{14}C]leucine and [^{14}C]valine, respectively, by the procedure of Rüdiger *et al.* (1972). The ^{14}C -labelled oxo acids were found to be free of the corresponding ^{14}C -labelled amino acids when examined by t.l.c. (Rüdiger *et al.*, 1972). All other reagents and materials were of the highest purity commercially available. The results in the Figures are representative of at least three essentially identical experiments. Other results are expressed as means $\pm$ S.E.M. All results are expressed per g wet wt. of heart.

Results

Fig. 1 shows the time course of production of $^{14}\text{CO}_2$ and 4-methyl-2-oxo[^{14}C]pentanoate from a rat heart infused with [^{14}C]leucine (0.1 mM). $^{14}\text{CO}_2$ production increased progressively during the perfusion, whereas 4-methyl-2-oxo[^{14}C]pentanoate production fell, suggesting that activation of the branched-chain 2-oxo acid dehydrogenase was occurring. This was confirmed by measurement of extractable branched-chain 2-oxo acid dehydrogenase activity in freeze-clamped hearts; after 10 min of perfusion, the extractable activity was $98 \pm 7.8 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, whereas after 40 min of perfusion the activity increased to $203.3 \pm 24.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (Table 1). Similar time-dependent activation of branched-chain 2-oxo acid dehydrogenase has been reported previously (Waymack *et al.*, 1980; Parker & Randle, 1980); since the intracellular 4-methyl-2-oxopentanoate concentration is probably one-fifteenth of the leucine concentration (Krebs & Lund, 1977), or less than $10 \mu\text{M}$, the activation of the enzyme complex may be analogous to that seen in substrate-free perfusion, where a similar gradual activation of the enzyme was seen (Waymack *et*

Fig. 1. Decarboxylation of [^{14}C]leucine and production of 4-methyl-2-oxo[^{14}C]pentanoate in the isolated perfused rat heart

Perfusion conditions were noted in the Experimental section. [^{14}C]Leucine was infused as indicated by the horizontal bar in the Figure. ●, $^{14}\text{CO}_2$ production; ▨, 4-methyl-2-oxopentanoate output. Leucine decarboxylation is defined as the rate of $^{14}\text{CO}_2$ production from infused [^{14}C]leucine via transamination and subsequent oxidative decarboxylation of the resultant 2-oxo acid. 4-Methyl-2-oxopentanoate output was determined by decarboxylation of perfusate samples with H_2O_2 , after the removal of dissolved $^{14}\text{CO}_2$ by acid treatment (Odessey & Goldberg, 1979).

Table 1. Effect of fatty acid on the activity of the branched-chain 2-oxo acid dehydrogenase complex in the perfused rat heart. Hearts were perfused in the presence of leucine (0.1 mM) under the conditions noted below before freeze-clamping and the measurement of the extractable branched-chain 2-oxo acid dehydrogenase activity.

Perfusion conditions	Branched-chain 2-oxo acid dehydrogenase activity (nmol·min ⁻¹ ·g ⁻¹)	No. of expts.
No addition, 15 min	98 ± 8	4
No addition, 40 min	203 ± 24	8
No addition, 10 min; octanoate (0.5 mM), 5 min	339 ± 27	4
No addition, 30 min; octanoate (0.5 mM), 10 min	356 ± 12	4
No addition, 30 min; glucose (5.5 mM), 10 min	135 ± 26	4
No addition, 30 min; glucose (5.5 mM), 10 min; glucose (5.5 mM) plus octanoate (0.5 mM), 10 min	178 ± 18	4
No addition, 30 min; palmitate (0.1 mM), 10 min	42 ± 9	4
No addition, 30 min; pyruvate (0.5 mM), 10 min	36 ± 7	4

Fig. 2. Effect of octanoate on the decarboxylation of [1-¹⁴C]leucine and production of 4-methyl-2-oxo[1-¹⁴C]-pentanoate in the perfused rat heart.

Perfusion conditions were described in the Experimental section. ●, ¹⁴CO₂ production; ○, 4-methyl-2-oxopentanoate output.

al., 1980). In agreement with this observation, at low 4-methyl-2-oxopentanoate concentrations (e.g. 5 μM), a similar time course of decarboxylation to that shown in Fig. 1 was obtained (results not shown), whereas at higher 4-methyl-2-oxopentanoate concentrations (e.g. 150 μM) no activation was seen (Buffington *et al.*, 1979), as the enzyme was already activated (Waymack *et al.*, 1980).

The effects of octanoate on ¹⁴CO₂ production from [1-¹⁴C]leucine (0.1 mM) were dependent on the duration of the perfusion before addition of the fatty acid. Infusion of octanoate (0.5 mM) after 10 min of [1-¹⁴C]leucine infusion led to a transient inhibition of ¹⁴CO₂ production, followed by a rapid elevation of flux to a new steady state (Fig. 2). On discontinuing the infusion of the fatty acid,

a rapid transient 'spike' of increased decarboxylation occurred, followed by a slower transient inhibition of flux. Production of 4-methyl-2-oxo[1-¹⁴C]pentanoate changed in the opposite direction to the changes in ¹⁴CO₂ production, suggesting again that modulation of the branched-chain 2-oxo acid dehydrogenase enzyme was responsible for this apparent change in the metabolic flux through this pathway. Consistent with this finding, at low (≤ 5 μM) concentrations of 4-methyl-2-oxo[1-¹⁴C]pentanoate essentially identical results were obtained (results not shown).

When hearts were perfused for 40 min before infusion of octanoate, a stable inhibition of ¹⁴CO₂ production was noted and was accompanied by an increased rate of 4-methyl-2-oxo[1-¹⁴C]pentanoate production (Fig. 3a). Similar results were seen in hearts perfused for 40 min with low (Fig. 3b) or high (results not shown) concentrations of 4-methyl-2-oxo[1-¹⁴C]pentanoate, and also in hearts perfused for 10 min with higher concentrations of 4-methyl-2-oxo[1-¹⁴C]pentanoate (≥ 10 μM) (see, e.g., Buffington *et al.*, 1979) or leucine (1 mM) (results not shown). Thus, under conditions where the branched-chain 2-oxo acid dehydrogenase activity was low (short perfusion times, low activator concentration) octanoate stimulated flux through the enzyme, whereas at higher concentrations of leucine or 4-methyl-2-oxopentanoate, or at longer perfusion times, permitting activation of the enzyme, inhibition of decarboxylation was observed. Measurement of extractable branched-chain 2-oxo acid dehydrogenase activity in freeze-clamped hearts showed that, under both conditions, octanoate activated the enzyme (Table 1); infusion after 10 min gave an activity of 338.6 ± 27.1 nmol·min⁻¹·g⁻¹, whereas after 40 min the extractable activity was 356.1 ± 11.5 nmol·min⁻¹·g⁻¹.

Fig. 3. Effect of octanoate on (a) [1-¹⁴C]leucine decarboxylation and effluent perfusate 4-methyl-2-oxo[1-¹⁴C]pentanoate and (b) decarboxylation of 4-methyl-2-oxo[1-¹⁴C]pentanoate in perfused rat hearts

Hearts were perfused for 30 min in the presence of unlabelled substrate before infusion of 1-¹⁴C-labelled substrate. Perfusion conditions were as noted in the Experimental section. ●, ¹⁴CO₂ production; ■, 4-methyl-2-oxopentanoate output.

Infusion of glucose (5.5 mM) caused a modest inhibition of ¹⁴CO₂ production from [1-¹⁴C]leucine, which was accompanied by an increase in 4-methyl-2-oxo[1-¹⁴C]pentanoate production (Fig. 4). Co-infusion of octanoate led to a further decrease in flux through the branched-chain 2-oxo acid dehydrogenase, and again output of branched-chain 2-oxo acid was increased. Measurement of the amount of active enzyme demonstrated that, in the presence of glucose, octanoate was unable to effect the large increase in branched-chain 2-oxo acid dehydrogenase activity seen in the absence of glucose, changing only from 134.7 ± 25.7 to 177.9 ± 18.4 nmol · min⁻¹ · g⁻¹, which was not significant.

Infusion of the long-chain fatty acid palmitate (0.1 mM) into hearts perfused with leucine (0.1 mM) provided results in marked contrast with those ob-

Fig. 4. Effects of infusion of glucose and octanoate on decarboxylation of [1-¹⁴C]leucine and effluent perfusate 4-methyl-2-oxo[1-¹⁴C]pentanoate

Hearts were perfused for 30 min in the presence of leucine (0.1 mM) before infusion of [1-¹⁴C]leucine. Perfusion conditions were as described in the Experimental section. ●, ¹⁴CO₂ production; ■, 4-methyl-2-oxopentanoate.

Fig. 5. Effect of palmitate on [1-¹⁴C]leucine decarboxylation and 4-methyl-2-oxo[1-¹⁴C]pentanoate output in the perfused rat heart

Hearts were perfused in the presence of leucine (0.1 mM) for 30 min before infusion of [1-¹⁴C]leucine. Palmitate was bound to bovine serum albumin before infusion. ●, ¹⁴CO₂ production; ■, 4-methyl-2-oxopentanoate output.

tained with octanoate (Fig. 5). Flux through the branched-chain reaction was inhibited by approx. 80%, with a concomitant increase in 4-methyl-2-oxo[1-¹⁴C]pentanoate output; Sans *et al.* (1980) observed similar results. Similar results were obtained with 4-methyl-2-oxo[1-¹⁴C]pentanoate at both high and low concentrations (results not shown). The onset of inhibition of flux by palmitate was considerably slower than that obtained with pyruvate, which inhibited leucine decarb-

Fig. 6. Effect of pyruvate on $[1-^{14}\text{C}]$ leucine decarboxylation and 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate output in the perfused rat heart

Hearts were perfused for 30 min in the presence of leucine (0.1 mM) before infusion of $[1-^{14}\text{C}]$ leucine. Perfusion conditions were as noted in the Experimental section. ●, $^{14}\text{CO}_2$ production; ▨, 4-methyl-2-oxopentanoate output.

oxylation by 90% (Fig. 6). Since activation of the long-chain fatty acid is necessary for entry into the mitochondrion, this process may be rate-limiting. Both palmitate and pyruvate caused almost complete inactivation of the branched-chain 2-oxo acid dehydrogenase, giving activities of 42.4 ± 9.0 and $36.3 \pm 6.7 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ respectively (Table 1).

Discussion

Several contradictory experimental observations have been reported concerning the interactions of fatty acid metabolism with the oxidation of branched-chain amino acids in muscle tissue (Buse *et al.*, 1972; Paul & Adibi, 1976; Buffington *et al.*, 1979; Sans *et al.*, 1980; Spydevold & Hokland, 1981). The present study was initiated to reassess the effects of fatty acids on branched-chain amino acid metabolism in the perfused rat heart. The concentrations of fatty acids infused into hearts in this study were chosen to be representative of physiological fatty acid concentrations observed during starvation and fat-feeding (Bach *et al.*, 1977).

Of great interest were the widely differing effects of the medium-chain fatty acid octanoate and the long-chain fatty acid palmitate on the regulation of branched-chain amino and 2-oxo

acids. Palmitate caused a large inhibition of flux, which was slow in onset, perhaps because activation of the fatty acid is rate-limiting. The inhibition of flux was accompanied by a dramatic decrease in the extractable activity of branched-chain 2-oxo acid dehydrogenase, from 203 to $42 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (Table 1). The effects of octanoate, however, were more complicated; octanoate infusion led to a large activation of the branched-chain 2-oxo acid dehydrogenase after both short and long intervals of perfusion with 0.1 mM-leucine (Table 1), but the effect of octanoate on production of $^{14}\text{CO}_2$ and 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate was dependent on the experimental conditions used. Under conditions where the branched-chain 2-oxo acid dehydrogenase activity was low initially (i.e. low activator concentration, short perfusion time), infusion of octanoate caused an increased flux through the enzyme complex (Fig. 2). However, under conditions favouring branched-chain 2-oxo acid dehydrogenase activation (i.e. high activator concentration and/or longer perfusion time), octanoate inhibited $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ leucine (Fig. 3a) or 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate (Fig. 3b). These results suggest that the regulatory effects of octanoate infusion on the metabolism of branched-chain amino and 2-oxo acids reflect two opposing actions of the fatty acid: firstly, feedback inhibition of the branched-chain 2-oxo acid dehydrogenase by elevated NADH/NAD⁺ and acyl-CoA/CoA ratios (Pettit *et al.*, 1978; Parker & Randle, 1978a,b; Danner *et al.*, 1978), and secondly, conversion of the enzyme complex into the active form, probably by dephosphorylation of the phosphoenzyme. Of interest is the report by Paxton & Harris (1983) demonstrating a 40% inhibition of isolated rabbit liver branched-chain 2-oxo acid dehydrogenase kinase activity by octanoate (200 μM). The octanoate-mediated activation of branched-chain 2-oxo acid dehydrogenase observed in the present experiments may reflect a direct inhibitory effect of the fatty acid on the inactivating kinase. Thus, when the enzyme activity is low, the net result of a large activation of the enzyme is more than enough to compensate for the feedback inhibition caused by NADH and acyl-CoA (Fig. 2), but, when the enzyme complex is already largely active, the feedback inhibition becomes more important, and net inhibition is seen (Fig. 3). At elevated 4-methyl-2-oxopentanoate concentrations (e.g. 150 μM), the branched-chain 2-oxo acid dehydrogenase is already maximally activated (Waymack *et al.*, 1980), and so the only effect of octanoate is a large inhibition of flux, e.g. almost 95% (Buffington *et al.*, 1979; Waymack *et al.*, 1980).

Also of note is that glucose largely prevents the activation of branched-chain 2-oxo acid dehydro-

genase by octanoate (Table 1), and under these conditions again an inhibition of flux is observed upon infusion of the fatty acid (Fig. 4). Although the results presented in this paper concern exclusively leucine metabolism, essentially identical results were obtained when leucine was replaced by valine, which is metabolized, at least initially, by the same enzyme systems as leucine (results not shown).

The effects of octanoate on the branched-chain 2-oxo acid dehydrogenase are in contrast with octanoate effects on the regulation of pyruvate dehydrogenase (EC 1.2.4.1), which in the rat heart is inhibited by approx. 95% by 0.76 mM-octanoate (Olson *et al.*, 1978). In the perfused liver, at low pyruvate concentrations both octanoate and oleate stimulate pyruvate decarboxylation by production of intramitochondrial acetoacetate and hence enhance pyruvate entry into the mitochondrion by exchange on the monocarboxylate transporter (Scholz *et al.*, 1978; Zwiibel *et al.*, 1982). However, in the heart, fatty acid oxidation does not result in ketogenesis, and an extensive inhibition of pyruvate dehydrogenase is observed (Olson *et al.*, 1978).

The results reported here are difficult to reconcile with those of Buse *et al.* (1972), who found stimulation of leucine oxidation by octanoate in both the presence and the absence of glucose, and saw no effect of palmitate on leucine decarboxylation. These differences may reflect the use of a small-volume recirculating perfusion system by Buse *et al.* (1972), with potential build-up of metabolic intermediates such as lactate and pyruvate, and consequent feedback inhibition of substrate metabolism. This possibility is supported by the very low rate of conversion of [1- 14 C]leucine into $^{14}\text{CO}_2$ reported by Buse *et al.* (1972); in the absence of glucose, 0.1 mM-[1- 14 C]leucine resulted in a $^{14}\text{CO}_2$ production rate of $81.6 \pm 8.4 \text{ nmol} \cdot 30 \text{ min}^{-1} \cdot \text{g}^{-1}$, whereas in the present study, a rate of $701 \pm 54 \text{ nmol} \cdot 30 \text{ min}^{-1} \cdot \text{g}^{-1}$ ($n = 8$) was obtained. Sans *et al.* (1980) found a decarboxylation rate of $1040 \pm 120 \text{ nmol} \cdot 30 \text{ min}^{-1} \cdot \text{g}^{-1}$ at 0.2 mM-[1- 14 C]leucine. It is likely that the experimental conditions used by Buse *et al.* (1972) maintained a low branched-chain 2-oxo acid dehydrogenase activity similar to that observed in Fig. 2(a) before octanoate infusion. In this case the octanoate-mediated activation of the enzyme might outweigh the inhibitory effects of the increased NADH/NAD $^{+}$ and acyl-CoA/CoA ratios.

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