Acute regulation of the branched-chain 2-oxo acid dehydrogenase complex by adrenaline and glucagon in the perfused rat heart

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Rates of transamination and decarboxylation of [1-¹⁴C]leucine at a physiological concentration (0.1 mM) were measured in the perfused rat heart. In hearts from fasted rats, metabolic flux through the branched-chain 2-oxo acid dehydrogenase reaction was low initially, but increased gradually during the perfusion period. The increase in ¹⁴CO₂ production was accompanied by an increase in the amount of active branched-chain 2-oxo acid dehydrogenase complex present in the tissue. In hearts from rats fed *ad libitum*, extractable branched-chain dehydrogenase activity was low initially, but increased rapidly during perfusion, and high rates of decarboxylation were attained within the first 10 min. Infusion of glucagon, adrenaline, isoprenaline, or adrenaline in the presence of phentolamine all produced rapid, transient, inhibition (40–50 %) of the formation of 4-methyl-2-oxo[1-¹⁴C]pentanoate and ¹⁴CO₂ within 1–2 min, but the specific radioactivity of 4-methyl-2-oxo[¹⁴C]pentanoate released into the perfusate remained constant. Glucagon and adrenaline infusion also resulted in transient decreases (16–24 %) in the amount of active branched-chain 2-oxo acid dehydrogenase. In hearts from fasted animals, infusion for 10 min of adrenaline, phenylephrine, or adrenaline in the presence of propranolol, but not infusion of glucagon or isoprenaline, stimulated the rate of ¹⁴CO₂ production 3-fold, and increased 2-fold the extractable branched-chain 2-oxo acid dehydrogenase activity. These results demonstrate that stimulation of glucagon or β -adrenergic receptors in the perfused rat heart causes a transient inhibition of branched-chain amino acid metabolism, whereas α -adrenergic stimulation causes a slower, more sustained, enhancement of branched-chain amino acid metabolism. Both effects reflect interconversion of the branched-chain 2-oxo acid dehydrogenase complex between active and inactive forms. Also, these studies suggest that the concentration of branched-chain 2-oxo acid available for decarboxylation can be regulated by adrenaline and glucagon.

INTRODUCTION

The initial steps in the catabolism of leucine, isoleucine and valine in mammalian tissues include reversible transamination to the 2-oxo acid, followed by oxidative decarboxylation. The latter step, catalysed by the branched-chain 2-oxo acid dehydrogenase complex, is rate-limiting to the pathway in extrahepatic tissues. The branched-chain dehydrogenase complex purified from liver or kidney is subject to feedback regulation by NADH and by the acyl-CoA products of the reaction [1–3]. Accordingly, conditions which result in increased ratios of NADH/NAD⁺ or acyl-CoAs/CoASH in the perfused heart, such as infusion of fatty acids, ketone bodies, glucose or pyruvate, also lead to inhibition of the metabolic flux through the branched-chain 2-oxo acid dehydrogenase reaction [4–8].

The branched-chain dehydrogenase complex can be interconverted between active (dephosphorylated) and inactive (phosphorylated) forms in the heart [9–12] and other tissues [13–15]. Covalent modification is accomplished by an associated protein kinase and phosphoprotein phosphatase, the activities of which also are regulated by various effectors [16–18].

Glucagon and adrenaline exert substantial acute effects on both the contractility and metabolic characteristics of cardiac tissue. Stimulation of perfused hearts with either of these hormones causes immediate release of tissue reserves of triacylglycerol [19,20] and glycogen [21–23], accompanied by acceleration of the oxidation of fatty acids [24,25], glucose [25,26] and pyruvate [25,27,28]. The influence of acute-acting hormones on

protein and amino acid metabolism in heart tissue has received comparatively little attention. Adrenaline was found to inhibit transiently the activity of the branched-chain 2-oxo acid dehydrogenase complex in perfused liver [29], and to depress the metabolism of leucine and valine in diaphragm muscle [30]. A single report has appeared on the effects of adrenaline and glucagon on branched-chain amino acid metabolism in rat heart, studied with a recirculating perfusion system [26]. Adrenaline stimulation for a total of 45 min resulted in a net increase in the amount of leucine oxidized during the final 30 min of perfusion. Under similar experimental conditions, glucagon was stimulatory in hearts from fed animals only when glucose was included, whereas in hearts from fasted rats stimulation occurred only in the absence of glucose.

In the experiments reported here, the acute regulatory effects of adrenaline and glucagon on the metabolism of branched-chain amino acids in rat heart have been investigated by the use of a non-recirculating perfusion apparatus. This experimental system permitted the observation of rapid, transient, hormonal effects in myocardial leucine metabolism which have not been reported previously. The extent to which activation/inactivation of the branched-chain 2-oxo acid dehydrogenase complex contributes to hormonal regulation of the pathway is discussed.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–175 g; from Houston facility of Harlan Sprague–Dawley, Indianapolis, IN,

U.S.A.) were given autoclaved diet (LM485; Teklad, Winfield, IN, U.S.A.) *ad libitum* and were maintained on a 07:00–21:00 h light cycle for 4–9 days. Chow was removed from the cages of fasting animals 2 days before they were killed. Non-recirculating Langendorff perfusion with Krebs–Henseleit buffer (1.25 mM- Ca^{2+}) containing the unlabelled substrate was established by standard methods [8]. For measurement of metabolic flux rates, infusion of $1\text{-}^{14}\text{C}$ -labelled substrate (1000 d.p.m./nmol) was initiated after 5 min of perfusion. Samples of the effluent perfusate (4 ml) were acidified with 0.5 ml of 2 M-sodium acetate, pH 3.3 [8], and $^{14}\text{CO}_2$ was trapped in phenethylamine and measured [4]. Radioactive 2-oxo acids in perfusate samples were determined by quantitative decarboxylation with H_2O_2 [31,32] and collection of the liberated $^{14}\text{CO}_2$ in the same way. Background corrections were made for the amount of ^{14}C label trapped after acidification or H_2O_2 treatment of a sample of the influent perfusate. Rates of $^{14}\text{CO}_2$ and 4-methyl-2-oxo[$1\text{-}^{14}\text{C}$]pentanoate formation, in nmol/min per g wet wt., were calculated from the volatile radioactivity trapped per sample, the sample volume, coronary flow rate, and the specific radioactivity of the substrate.

For specific-radioactivity measurements, the concentration of 4-methyl-2-oxopentanoate was determined by conversion into the quinoxalinol derivative, and separation by reversed-phase h.p.l.c. Published procedures [33,34] were adapted to minimize sample dilution and to simplify chromatographic conditions. Sample (150 μl) was mixed with 30 μl of 0.06 M-*o*-phenylenediamine in 12 M-HCl and heated at 80 °C in the dark for 30 min. Immediately after adjustment to pH 5 with 90 μl of 10 M-potassium acetate, a 200 μl portion was injected on to an octadecylsilane column (Spherisorb 5 μm , from Custom LC, Houston, TX, U.S.A.). Separation was achieved isocratically with acetonitrile/aq. 0.025 M-ammonium phosphate, pH 7.0 (7:13, v/v). Fluorescence was monitored at 420 nm, with excitation at 340 nm, by using a Waters model 420 detector. Typical elution times were 5.3 min for pyruvate, 12.4 min for 3-methyl-2-oxobutyrates, 14.0 min for 4-methyl-2-oxopentanoate and 18.0 min for 3-methyl-2-oxopentanoate. External standards were prepared with each sample set by adding 4-methyl-2-oxopentanoate to perfusate. The integrated peak area was proportionate up to 3 μM .

The amount of active branched-chain 2-oxo acid dehydrogenase in the cardiac tissue was determined within 2 days after freeze-clamping as previously described [35]. The assay gave a linear progress curve for the enzymic reaction after subtraction of a background correction (20–25%) determined in identical samples but with zero incubation time. The yield of acid-precipitable non-collagen protein in homogenate samples [36,37] was 55 ± 2 mg/g of tissue (wet wt.) for fed rats, and 58 ± 1 mg/g for fasted animals.

Figures (except Fig. 2) show the results of representative experiments. Each experiment was repeated independently at least three times, with nearly identical outcome. Where large numbers of observations were compiled (Fig. 2, Tables 1 and 2), a complete-block experimental design was employed. Average values given in the text or in Tables are expressed $\pm$ S.E.M. Statistical analyses employed Student's two-tailed *t* test.

L-[$1\text{-}^{14}\text{C}$]Leucine and L-[$1\text{-}^{14}\text{C}$]valine (50 Ci/mol) were purchased from ICN (Irvine, CA, U.S.A.). 4-Methyl-2-

Fig. 1. Radioactive metabolites formed by rat hearts perfused with 0.1 mM-leucine

[$1\text{-}^{14}\text{C}$]Leucine was introduced at 5 min, and the rates of formation of $^{14}\text{CO}_2$ ($\circ$), 4-methyl-2-oxo[$1\text{-}^{14}\text{C}$]pentanoate ($\bullet$), and the sum of both metabolites ($\blacksquare$) were determined from the amounts released into the perfusate. (a) Hearts from rats fed *ad libitum*; (b) hearts from rats fasted for 2 days.

oxo[$1\text{-}^{14}\text{C}$]pentanoate was synthesized enzymically [38] and stored in 1 mM-HCl at -20 °C. L-Adrenaline (Sigma, St. Louis, MO, U.S.A.) and crystalline glucagon (Eli Lilly, Indianapolis, IN, U.S.A.) were dissolved in 1–2 mM-HCl. Other reagents were obtained from the following sources: L-isoprenaline D-bitartrate, L-phenylephrine hydrochloride, DL-propranolol hydrochloride and *o*-phenylenediamine dihydrochloride (Sigma); phentolamine hydrochloride (CIBA-Geigy, Summit, NJ, U.S.A.); dichloroacetic acid (Fluka Chemical Corp., Hauppauge, NY, U.S.A.), and BDH Chemicals, Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

Infusion of [$1\text{-}^{14}\text{C}$]leucine at a physiological concentration into the isolated perfused rat heart resulted in the rapid formation and release of 4-methyl-2-oxo[$1\text{-}^{14}\text{C}$]pentanoate and $^{14}\text{CO}_2$ into the perfusate within 5 min (Fig. 1). In hearts obtained from rats fed *ad libitum* (Fig. 1a), most of the 2-oxo acid formed by transamination was decarboxylated, leaving low residual amounts of 4-methyl-2-oxo[$1\text{-}^{14}\text{C}$]pentanoate. In contrast, when hearts

Table 1. Rate of increase in extractable branched-chain 2-oxo acid dehydrogenase activity during perfusion of rat hearts with 0.1 mM-leucine

Rat hearts were perfused with 0.1 mM-leucine for the indicated times, freeze-clamped, and assayed for active branched-chain 2-oxo acid dehydrogenase complex as described in the Materials and methods section. Values are given as means $\pm$ S.E.M. Numbers in parentheses indicate the numbers of hearts per treatment group. * The difference between the values for the fed and fasted treatment groups is statistically significant at $P < 0.01$ (two-tailed t test).

Perfusion time	Activity (nmol of $^{14}\text{CO}_2$ /min per mg of protein)	
	Fed rats	Fasted rats
0 s	0.29 ± 0.08 (4)	0.24 ± 0.15 (3)
10 s	0.32 ± 0.12 (4)	0.26 ± 0.12 (4)
2 min	0.76 ± 0.41 (4)	0.34 ± 0.04 (4)
10 min	1.50 ± 0.15 (10)	0.45 ± 0.17 (6)
20 min	2.11 ± 0.18 (11)	0.71 ± 0.17 (7)
30 min	2.54 ± 0.19 (11)	1.11 ± 0.29 (9)
5 min, dichloroacetate	3.60 ± 0.30 (4)	2.80 ± 0.63 (4)

Fig. 2. Correlation between metabolic flux and extractable branched-chain 2-oxo acid dehydrogenase activity

Hearts from fasted rats ($\circ$, $\square$, $\triangle$) or fed rats ($\bullet$, $\blacksquare$, $\blacktriangle$) were perfused under the conditions shown in Fig. 1 for 10 min ($\circ$, $\bullet$), 20 min ($\square$, $\blacksquare$), or 30 min ($\triangle$, $\blacktriangle$). Immediately after collection of perfusate samples for measurement of the rate of decarboxylation of 0.1 mM- $[1-^{14}\text{C}]$ leucine, the hearts were freeze-clamped and the amount of active branched-chain dehydrogenase complex was determined. The mean values $\pm$ S.E.M. for each treatment group (three to eight individuals per group) are plotted. The least-squares line fitting a total of 35 observations is shown (regression coefficient = 0.94, slope = 0.24). Analysis of variance verified that there was no significant deviation from linearity.

were obtained from fasted rats (Fig. 1b), only a small fraction of the 4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate underwent decarboxylation during short perfusion intervals. As the perfusion continued, however, the flux through the decarboxylation step increased steadily. Similar

results were obtained with 0.1 mM- $[1-^{14}\text{C}]$ valine or 0.01 mM-4-methyl-2-oxo $[1-^{14}\text{C}]$ pentanoate in place of 0.1 mM-leucine as the labelled substrate.

The rate at which $^{14}\text{CO}_2$ formation increased spontaneously in this manner was subject to day-to-day as well as rat-to-rat variability, and care should be taken in comparison of values obtained in separate experiments. Nevertheless, as the results that follow show, data obtained in replicate perfusions performed on a given day were consistent, allowing statistically meaningful comparisons among experimental groups.

The amount of active branched-chain 2-oxo acid dehydrogenase complex measured in extracts of freeze-clamped, unperfused, heart tissue was low in both fed and fasted rats (Table 1), in agreement with the observations of other groups [8,11,39–41]. During subsequent perfusion, the extractable activity present in hearts from fed animals increased rapidly, whereas in hearts from fasted animals the activation occurred more slowly. Dichloroacetate inhibits the branched-chain 2-oxo acid dehydrogenase kinase [42], and in the perfused heart results in activation of the complex to maximum values [7,9,42]. The amount of activity extracted after perfusion with 1 mM-dichloroacetate was the same in hearts obtained from either fed or fasted animals, indicating that the total branched-chain 2-oxo acid dehydrogenase complex in the tissue was not affected appreciably by 2 days of food deprivation, a conclusion also reached by other laboratories [39,41,43]. It is likely that the difference in the metabolic flux through the decarboxylation step observed in perfused hearts upon fasting (Fig. 1) reflects this difference in the rates at which the inactive complex was converted into the active form during perfusion with 0.1 mM-leucine. Furthermore, within a series of replicate perfusions, the extractable activity was found to be directly proportional to the rate of flux measured just before freeze-clamping (Fig. 2).

The slower rate of spontaneous activation in hearts from fasted animals during the early stages of perfusion suggested that fasting may influence the activity of the associated branched-chain 2-oxo acid dehydrogenase kinase or phosphatase. Indeed, an increase in the activity of this protein kinase in heart mitochondrial extracts after fasting for 48 h was reported recently [44]. It seems

Fig. 3. Effect of adrenaline on metabolism of 0.1 mM-[1- 14 C]-leucine to $^{14}\text{CO}_2$ (○) and to 4-methyl-2-oxo[1- 14 C]pentanoate (●) in hearts from fed rats (a) or fasted rats (b)

'Fractional decarboxylation' (■) denotes the ratio of $^{14}\text{CO}_2/^{14}\text{CO}_2 + 4\text{-methyl-2-oxo[1-}^{14}\text{C]pentanoate}$.

Fig. 4. Effect of glucagon on conversion of 0.1 mM-[1- 14 C]leucine into $^{14}\text{CO}_2$ (○) and 4-methyl-2-oxo[1- 14 C]pentanoate (●) in hearts from fed rats (a) or fasted rats (b)

Fractional decarboxylation (■) denotes the ratio of $^{14}\text{CO}_2/^{14}\text{CO}_2 + 4\text{-methyl-2-oxo[1-}^{14}\text{C]pentanoate}$.

reasonable that the observed differences in the regulatory properties of the enzymes may be related to the elevation of the amounts of fatty acids in heart tissue during short-term fasting [45]. Long-chain fatty acids were shown to prevent spontaneous activation of the complex under perfusion conditions similar to the present study [6].

The availability of experimental conditions wherein decarboxylation of the 2-oxo acid occurs with high efficiency (fed animals) or low efficiency (fasted animals) provided the opportunity to observe both stimulatory and inhibitory regulatory effects of adrenaline on myocardial leucine metabolism (Fig. 3). In hearts from fed animals (Fig. 3a) adrenaline infusion resulted in a rapid, transient, decrease in the rate of formation of $^{14}\text{CO}_2$. In replicate experiments performed on a single day, the flux through the decarboxylation step was inhibited maximally ($41 \pm 3\%$ decrease, $n = 4$) at 1.5–2 min after introduction of the agonist, then returned to the normal value over a period of 8–10 min. Likewise,

a rapid, transient, decrease in 4-methyl-2-oxo[^{14}C]pentanoate also was observed, except that maximal inhibition ($37 \pm 4\%$, $n = 4$) occurred sooner, 0.5–1 min after adrenaline infusion began, and returned to control values by 2–3 min of infusion. Because the changes in 4-methyl-2-oxo[^{14}C]pentanoate amounts preceded those in $^{14}\text{CO}_2$, there was a transient decrease in the fraction of the 2-oxo acid formed from leucine that underwent decarboxylation, as estimated by the ratio $^{14}\text{CO}_2/^{14}\text{CO}_2 + 4\text{-methyl-2-oxo[1-}^{14}\text{C]pentanoate}$ (Fig. 3, upper trace). In other words, efficiency of utilization of 2-oxo acid was diminished upon the infusion of hormone.

Similar rapid decreases in the formation of 4-methyl-2-oxo[^{14}C]pentanoate and $^{14}\text{CO}_2$ were observed in hearts from fasted rats (Fig. 3b). But, in addition, stimulation of the rate of decarboxylation was noted after 10 min of adrenaline infusion. The formation of $^{14}\text{CO}_2$ increased from 2.9 ± 0.8 at 15 min to 12.1 ± 2.7 nmol/min per g at

Table 2. Effects of adrenaline and glucagon on amount of branched-chain 2-oxo acid dehydrogenase activity in perfused rat hearts

Rat hearts were perfused with buffer containing 0.1 mM-leucine for 15 min. At that time, hormone infusion was initiated in the experimental groups, and perfusion was allowed to continue for the specified length of time before freeze-clamping. Controls were freeze-clamped at the same times, but no hormone was infused. The frozen tissues were assayed for active branched-chain 2-oxo acid dehydrogenase complex as described in the Materials and methods section. Values are given as means $\pm$ S.E.M., and numbers in parentheses denote the number of hearts per treatment group. Levels of statistical significance (*P* value) for comparisons between corresponding control and experimental values were evaluated by the two-tailed Student's *t* test: 'ns' denotes a lack of statistical significance.

		Branched-chain dehydrogenase activity (nmol of $^{14}\text{CO}_2$ /min per mg of protein)					
		Control	Adrenaline	<i>P</i> value	Control	Glucagon	<i>P</i> value
Fed	1½ min	2.41 $\pm$ 0.11 (24)	1.83 $\pm$ 0.20 (16)	< 0.01	1.61 $\pm$ 0.09 (19)	1.36 $\pm$ 0.07 (14)	< 0.05
	3½ min	2.72 $\pm$ 0.26 (6)	2.50 $\pm$ 0.29 (6)	ns	2.08 $\pm$ 0.08 (7)	1.86 $\pm$ 0.12 (7)	ns
	10 min	2.54 $\pm$ 0.14 (15)	2.62 $\pm$ 0.18 (17)	ns	2.12 $\pm$ 0.16 (11)	2.34 $\pm$ 0.26 (11)	ns
Fasted	1½ min	0.29 $\pm$ 0.05 (16)	0.31 $\pm$ 0.04 (17)	ns	0.44 $\pm$ 0.06 (16)	0.37 $\pm$ 0.04 (16)	ns
	10 min	0.38 $\pm$ 0.06 (14)	0.82 $\pm$ 0.07 (15)	< 0.001	0.48 $\pm$ 0.07 (15)	0.66 $\pm$ 0.06 (17)	< 0.05

25 min of perfusion when adrenaline was infused over this interval ($n = 9$), compared with an increase from 2.1 ± 0.4 to 4.1 ± 1.0 nmol/min per g in control hearts ($n = 10$). This 3-fold stimulation of flux upon infusion of adrenaline into hearts from fasted rats was statistically significant ($P < 0.01$).

The influence of agents specific for α - and β -adrenergic receptors on leucine metabolism was investigated in hearts from fasted rats, with the following results (not shown). The transient inhibitory effect of adrenaline could be blocked by propranolol, a β -antagonist, whereas isoprenaline, a β -agonist, was able to elicit the transient inhibition. Conversely, the stimulatory effect of adrenaline on branched-chain 2-oxo acid dehydrogenase flux was blocked by phentolamine, an α -antagonist, whereas infusion of phenylephrine, an α -agonist, evoked the stimulation of decarboxylation. Hence it appeared likely that the two metabolic responses to adrenaline in the fasted rat heart, i.e. transient flux inhibition followed by stimulation of flux, were mediated separately by β - and α -adrenergic receptors respectively.

Upon glucagon infusion into hearts from fed rats, as with adrenaline, the rates of formation of both 4-methyl-2-oxo[1- ^{14}C]pentanoate and $^{14}\text{CO}_2$ were inhibited rapidly and transiently (Fig. 4a). In replicate experiments, the decarboxylation rate was found to be inhibited maximally by $46 \pm 2\%$ ($n = 4$) after 1–1.5 min of glucagon stimulation, then returned to control values at 5–8 min. The greatest decrease in formation of 4-methyl-2-oxo[1- ^{14}C]pentanoate ($45 \pm 2\%$, $n = 4$) was reached at 0.5–1 min of infusion, with return to control values by 2–3 min. As with adrenaline, the changes in 4-methyl-2-oxo[1- ^{14}C]pentanoate were more rapid than the changes in $^{14}\text{CO}_2$, such that the fraction of 4-methyl-2-oxopentanoate being decarboxylated in the tissue underwent a transient decrease upon glucagon stimulation.

In hearts from fasted rats (Fig. 4b), the infusion of glucagon resulted in similar transient decreases in the rates of formation of $^{14}\text{CO}_2$ and of 4-methyl-2-oxo[1- ^{14}C]pentanoate. The increase in the rate of leucine decarboxylation during glucagon infusion (from 1.2 ± 0.1 nmol/min per g at 15 min of perfusion to 4.1 ± 0.7 nmol/min per g at 25 min; $n = 10$) did not differ

significantly from that occurring spontaneously in the absence of glucagon (from 1.3 ± 0.1 to 3.8 ± 0.6 nmol/min per g; $n = 10$). With respect to leucine metabolism, then, the effects of glucagon were very similar to those seen upon β -adrenergic stimulation of the perfused heart. In hearts from either fed or fasted rats, we observed no differences in these flux changes in response to glucagon upon addition of 5.5 mM-glucose to the perfusion medium.

To assess the degree to which conversion of the branched-chain 2-oxo acid dehydrogenase complex activity contributed to the observed regulatory effects of the hormones on metabolic flux, extractable activity of the complex was measured in tissue from hearts that were freeze-clamped during infusion of adrenaline or glucagon (Table 2). The mean extractable activity in hearts from fed animals after only 90 s of adrenaline infusion decreased by 24% compared with the control group ($P < 0.01$). Likewise, when glucagon was infused for 90 s, the extractable activity of the enzyme complex decreased by 16% ($P < 0.05$). In both cases, this rapid inactivation of the complex in response to exposure to adrenaline or glucagon was also reversed rapidly, with the extractable activity returning to control values by 4 min of hormone infusion.

Similar measurements in hearts from fasted animals showed a significant ($P < 0.001$), approx. 2-fold, increase in the amount of active enzyme after 10 min of adrenaline infusion, compared with control fasted-rat hearts. Each of these hormone-induced changes in branched-chain 2-oxo acid dehydrogenase activity appeared to give rise to a corresponding perturbation in [^{14}C]leucine metabolic flux. On the other hand, in fasted rat hearts which had been treated with glucagon for 10 min, a modest (40%) increase in extractable activity was observed. Significant stimulation of flux through the decarboxylation step had not been observed under the same experimental conditions. However, experimental variability would make it difficult to measure a small flux change over this period.

The involvement of enzyme inactivation in the transient inhibitory effects of these hormones upon initiation of leucine metabolism was further examined in

Fig. 5. Influence of hormones on the concentration of 4-methyl-2-oxopentanoate in perfusate (●) and its specific radioactivity (■)

Hearts from rats fed *ad libitum* were perfused with 0.1 mM-[1- 14 C]leucine, and exposed to adrenaline (a) under the same conditions as in Fig. 3, or to glucagon (b) under the conditions as in Fig. 4. Perfusate samples were analysed for 4-methyl-2-oxo[1- 14 C]pentanoate and for total 4-methyl-2-oxopentanoate as described in the Materials and methods section.

the following perfusion experiment. Under conditions of hormone stimulation as in Figs. 3 and 4, but in the presence of 1 mM-dichloroacetate to inhibit inactivation of the branched-chain complex by the kinase, the rate of 4-methyl-2-oxo[1- 14 C]pentanoate release and of decarboxylation both underwent transient decreases upon infusion of adrenaline or glucagon, as had been observed in the absence of dichloroacetate. However, the changes in $^{14}\text{CO}_2$ production coincided with the decreases in 4-methyl-2-oxo[1- 14 C]pentanoate, such that the fraction of 4-methyl-2-oxopentanoate undergoing decarboxylation remained constant throughout hormone stimulation. Thus the prevention of inactivation of the branched-chain dehydrogenase complex also prevented the effects of adrenaline and glucagon on the efficiency of utilization of the 2-oxo acid substrate.

A transient decrease in the rate of decarboxylation during leucine infusion would be expected, if anything, to result in an increase in the release of 4-methyl-2-oxopentanoate into the perfusate, rather than the decrease observed upon stimulation with adrenaline or glucagon. Therefore the possibility was considered that

the hormones may in some way influence the isotopic dilution of the labelled substrate, for example by altering proteolytic rates in the tissue. Because leucine and 4-methyl-2-oxopentanoate are interconverted rapidly in the tissue, the degree of this isotopic dilution could be assessed by measuring the specific radioactivity of the 4-methyl-2-oxo[1- 14 C]pentanoate released into the perfusate. In control experiments similar to those in Fig. 1, the total perfusate concentration of 4-methyl-2-oxopentanoate was measured fluorimetrically, and compared with the amount of ^{14}C -labelled 2-oxo acid measured in the same samples by H_2O_2 treatment. After a 10 min period of isotopic equilibration, the specific radioactivity of 4-methyl-2-oxo[1- 14 C]pentanoate released into the perfusate by hearts from fed animals was $75 \pm 5\%$ ($n = 3$) of the specific radioactivity of the infused [1- 14 C]leucine, and remained constant over the next 40 min of perfusion. Likewise, when hearts from fasted animals were perfused, the specific radioactivity of perfusate 4-methyl-2-oxo[1- 14 C]pentanoate remained at $77 \pm 4\%$ ($n = 3$) relative to infused [1- 14 C]leucine, over the period from 10 to 50 min.

In order to determine whether the isotopic equilibria were perturbed by exposure to adrenaline or glucagon, the amounts of both ^{14}C -labelled and total 4-methyl-2-oxopentanoate were measured in samples of perfusate taken at 30 s intervals before and during the infusion of hormone (Fig. 5). It was found that the total perfusate 4-methyl-2-oxopentanoate concentration changed in parallel with ^{14}C -labelled 4-methyl-2-oxopentanoate, such that the specific radioactivity of the compound remained essentially unchanged throughout the periods of adrenaline or glucagon infusion. Thus there was no evidence of any appreciable hormone-stimulated changes in the intracellular leucine pool of the heart. Instead, the present experimental results demonstrated substantial effects of adrenaline and glucagon on the concentration of 4-methyl-2-oxopentanoate available to the branched-chain dehydrogenase complex. Although intramitochondrial 2-oxo acid concentrations are not known, the tissue 4-methyl-2-oxopentanoate concentration is low ($< 4 \mu\text{M}$) [46,47] compared with the K_m for this substrate (10–50 μM) [1,3,12]. Given the sub-saturating substrate concentration, it follows that the change in 2-oxo acid availability should contribute to the transient decrease in the rate of flux of branched-chain compounds through the decarboxylation step.

Several mechanisms can be suggested by which 4-methyl-2-oxopentanoate amounts might be controlled by adrenaline or glucagon. These include regulation of the transport of amino acid or 2-oxo acid across the sarcolemma or mitochondrial membrane, or, alternatively, transient inhibition of the transamination of the amino acid. The data at present available do not allow one to distinguish the actual means of hormonal regulation of 2-oxo acid concentration.

Our results may be compared with the early studies of perfused rat heart by Buse *et al.* [26], who determined that inclusion of adrenaline in a continuously recirculating perfusion medium brought about an increase in the amount of branched-chain amino acid decarboxylation over a 30 min period. Those observations appear consistent with the stimulation of leucine decarboxylation that occurred upon prolonged adrenaline infusion in the present study (Fig. 3b). In Buse's [26] experiments, hearts were exposed to the hormone for 15 min before addition

of the ^{14}C -labelled tracer. As a result, transient hormonal effects such as the inhibition of flux reported here would not have been detected in their system. Although we did not observe significant stimulation of flux during glucagon infusion (Fig. 4), a small degree of activation of the branched-chain enzyme complex did occur in hearts from fasted animals (Table 2). It is interesting that Buse *et al.* [26] noted that a small stimulation of flux in hearts from fasted rats occurred at low concentrations of glucagon (5–20 nM), and that this effect did not occur at 0.35 μM -glucagon in their system.

The outcome of the present study may be summarized briefly as follows. Stimulation of the heart with adrenaline or glucagon influenced dramatically the formation of the 2-oxo acid from leucine. These changes in the availability of substrate contributed to the transient decreases in metabolic flux through the decarboxylation reaction that occurred in response to hormone infusion. Modulation of the activity state of the branched-chain 2-oxo acid dehydrogenase complex represented a second mechanism by which adrenaline and glucagon served to regulate the catabolism of leucine. Both transient inactivation and slower, more sustained, activation of the complex were observed. The additional influence of other regulatory phenomena on the branched-chain 2-oxo acid dehydrogenase complex in this type of metabolic preparation, such as metabolite feedback, remain to be determined.

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