SUBCELLULAR DISTRIBUTION OF KEY METABOLITES IN ISOLATED LIVER CELLS FROM FASTED RATS

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1. Introduction

The knowledge of subcellular metabolite distribution is of fundamental importance for considerations on metabolic regulation. In previous studies [1,2] we have determined the cytosolic and mitochondrial phosphorylation state of adenine nucleotides in relation to pyruvate dehydrogenase regulation in isolated hepatocytes by a modification of the digitonin fractionation method of Zuurendonk and Tager [3]. In this paper we report on the compartmentation of key metabolites of the Krebs cycle, of gluconeogenesis and of ketogenesis.

2. Materials and methods

L(−)Carnitine chloride was kindly supplied by Otsuka Pharmaceutical Factory, Osaka, Japan. Enzymes and coenzymes were products of Boehringer, Mannheim, Germany. Other reagents were those used in earlier studies [1,2]. Liver cells were isolated from 24–48 h fasted male Sprague–Dawley rats, weighing 200–250 g (W. Gassner, Sulzfeld, Germany) and incubated as described [1] except that the isolation and incubation media were fortified with 1 mM carnitine [5] and that 0.9% defatted [4] bovine serum albumin was present in the incubation medium. After an incubation period of 10 min the total intracellular amount of metabolites was determined by centrifuging (10 s, Eppendorf centrifuge model 3200) the cells of 0.4 ml of the incubation mixture in an Eppendorf cup through 0.5 ml of a silicon oil mixture (AR 200/AR 20 = 3:1, 37°C) into 0.24 ml of 12% (w/v) $HClO_4$. Another portion of 0.2 ml was treated with digitonin and fractionated as described [2] except that calcium-free Krebs–Henseleit bicarbonate buffer, pH 7.4 was used instead of the sucrose containing morpholinopropane sulfonic acid buffer. Malate, citrate and CoASH were determined by standard methods [6–8] using a Perkin–Elmer double beam spectrophotometer. Ketone bodies were assayed according to [9,10], and acetyl-CoA was measured by the method of [11]. Oxaloacetate was determined by an isotope dilution test as described in a separate paper [12].

The carry over into the pellet of [^{14}C]oxaloacetate added to the digitonin medium (1.38 nmol) was 0.8%. During the digitonin procedure essentially no oxaloacetate was produced from 10 mM pyruvate or 0.1 mM malate.

The tabulated amounts of metabolites are given as mean values ± S.E.M. for the numbers of different cell preparations in parentheses.

3. Results and discussion

In the present study it was attempted to determine the cytosolic and mitochondrial concentrations of key metabolites of gluconeogenesis, ketogenesis and the citric acid cycle in isolated liver cells by the digitonin fractionation method [2,3]. Hepatocytes isolated from 24–48 h starved rats incubated for 10 min without exogenous substrate displayed levels of acetyl-CoA, CoASH and oxaloacetate well comparable with those found in freeze-clamped liver tissue from starved rats [12,13], while comparable levels of malate and citrate [14] were found only in cells incubated with lactate or pyruvate. On the basis of 10^8 cells corresponding to 1 g of fresh liver [15] or 0.29 ± 0.01 (n = 30) g dry wt., containing 0.4 ml

and 0.048 ml cytosolic [16] and mitochondrial water [17,18], respectively, the metabolite concentrations were calculated to be higher in the mitochondrial matrix than in the cytosol. CoASH was located almost exclusively in the mitochondrial fraction, yielding concentrations of 2.3–3 4 mM (table 1). Acetyl-CoA also displayed a high mitochondrial/cytosolic (= m/c) ratio, depending on the substrate used for cell incubation (table 2). The steepest gradient was observed in the presence of oleate, which caused a 3-fold

Table 1
Mitochondrial location of coenzyme A in isolated hepatocytes from fasted rats

Substrate added	mM	Amount (nmol/g dry wt.) Total	Pellet	Mit. conc. (mM)
None		390 ± 52 (7)	344 ± 53 (7)	2.4
Lactate	10	484 ± 114 (5)	484 ± 99 (5)	3.4
Oleate	0.9	312 ± 80 (6)	343 ± 82 (6)	2.4
Oleate + lactate	0.9 10	401 ± 87 (8)	375 ± 92 (8)	2.7
Pyruvate	7	428 ± 95 (5)	468 ± 54 (5)	3.3
Aspartate	7	300 ± 56 (8)	330 ± 60 (8)	2.3
Ethanol	12	510 ± 85 (7)	482 ± 84 (7)	3.4

Table 2
Subcellular distribution of acetyl-CoA in isolated hepatocytes from fasted rats in the presence of various substrates

Substrate added	mM	Amount (nmol/g dry wt.) Total	Pellet	(T − P)	Concentration (mM) Mit.	Cyt.	Concentration gradient (m/c)
None		215 ± 23 (14)	143 ± 13 (14)	72	0.86	0.052	16.5
Lactate	10	251 ± 16 (11)	142 ± 9 (11)	109	0.86	0.080	10.8
Oleate	0.9	389 ± 29 (11)	448 ± 29 (11)	–	2.74	–	–
Oleate + lactate	0.9 10	325 ± 20 (11)	262 ± 23 (11)	63	1.63	0.046	35.4
Pyruvate	7	441 ± 20 (13)	262 ± 12 (13)	179	1.63	0.131	12.7
Aspartate	7	239 ± 28 (14)	154 ± 13 (14)	85	0.94	0.063	14.9
Ethanol	12	420 ± 31 (11)	313 ± 20 (11)	107	1.88	0.078	24.1

Table 3
Ketone body production of hepatocytes in the presence of various substrates

Substrate added	mM	Ketone body production (μmol/10 min per g dry wt.) 3-Hydroxybutyrate	Acetoacetate	Total	3-Hydroxybutyrate/ acetoacetate
None		2.17 ± 0.57 (13)	20.0 ± 1.8 (13)	22.17	0.11
Lactate	10	2.90 ± 0.70 (10)	17.1 ± 1.7 (10)	19.91	0.17
Oleate	0.9	49.1 ± 6.1 (10)	70.1 ± 4.8 (10)	119.2	0.7
Oleate + lactate	0.9 10	40.9 ± 3.5 (13)	42.1 ± 2.0 (13)	83	0.97
Pyruvate	7	6.8 ± 0.8 (13)	36.1 ± 2.5 (13)	42.9	0.19
Aspartate	7	3.2 ± 0.6 (13)	28.9 ± 2.0 (13)	32.1	0.11
Ethanol	12	3.2 ± 0.8 (13)	31.5 ± 2.5 (13)	34.7	0.10

increase of the matricial concentration, while it became unmeasurable low in the cytosol. The stimulation by oleate of acetyl-CoA accumulation was counteracted by lactate. In the presence of pyruvate or ethanol the cells contained the same amounts of acetyl-CoA as with oleate, the subcellular distribution, however, was different as 25–40% were now located extra-mitochondrially. Consistent with studies on ketogenesis in isolated liver mitochondria [19,20], ketone body formation from different substrates by intact hepatocytes (table 3) did not correlate with the mitochondrial concentration of acetyl-CoA (table 2) but rather with the acetyl-CoA/CoSH ratio (fig.1).

The availability of oxaloacetate for citrate synthase has been considered the major regulatory factor in determining the flux of acetyl-CoA either into the Krebs cycle or into ketogenesis ([21,22], for a review see [23]). Data on the subcellular distribution of oxaloacetate are presented in table 4.

It appears that the mitochondrial oxaloacetate concentrations are higher than the K_M of liver citrate synthase of 2–7 μM [24–27], and about two orders of magnitude higher than those calculated on the basis of mass action equilibria [28–30]. While the matricial oxaloacetate level was kept fairly constant, marked changes occurred in the cytosol, which were highest in the cells incubated with lactate plus oleate. The reduction of mitochondrial oxaloacetate by about 25% by oleate would hardly explain the large increase of ketone body formation since ethanol which lowers matricial oxaloacetate to the same extent (see table 4) was much less ketogenic (see table 3). The possibility remains that oleate inhibits the entry

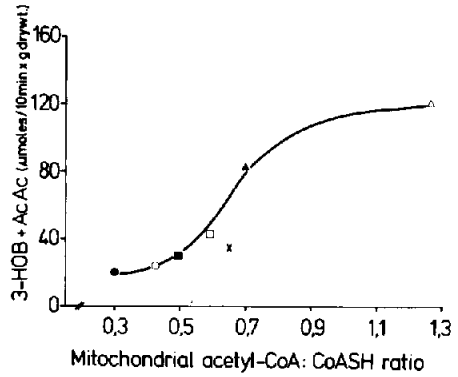

Fig.1. Ketone body formation by isolated liver cells from starved rats as a function of the mitochondrial acetyl-CoA: CoASH ratio. △, 0.9 mM oleate; ▲, 0.9 mM oleate plus 10 mM lactate; □, 7 mM pyruvate; ×, 12 mM ethanol; ■, 7 mM aspartate; ●, 10 mM lactate; ○, no addition.

Table 4
Subcellular distribution of oxaloacetate in isolated liver cells in the presence of various substrates

Substrate added	mM	Amount (nmol/g dry wt.) Total	Pellet	(T − P)	Concentration (μM) Mit.	Cyt.	Concentration gradient (m/c)
None		5.00 ± 0.4 (8)	2.1 ± 0.3 (8)	2.9	12.9	2.1	6.1
Lactate	10	12.7 ± 1.6 (7)	3.1 ± 0.4 (8)	9.6	18.7	7.0	2.7
Oleate	0.9	3.8 ± 0.5 (8)	1.6 ± 0.2 (8)	2.2	10.0	1.5	6.7
Oleate + lactate	0.9 10	28.9 ± 2.2 (5)	4.4 ± 0.3 (7)	24.5	27.1	17.9	1.5
Pyruvate	7	13.5 ± 1.7 (8)	2.7 ± 0.5 (8)	10.8	16.5	7.9	2.1
Aspartate	7	14.2 ± 1.1 (5)	4.4 ± 0.4 (5)	9.8	27.1	7.1	3.8
Ethanol	12	3.6 ± 0.5 (6)	1.5 ± 0.2 (6)	2.1	9.3	1.5	6.2

of acetyl-CoA into the Krebs cycle by rising the level of long-chain acyl CoA [2,31] which is known to increase the K_M of citrate synthase for oxaloacetate several fold [26]. Consistent with this view is the observation, that the cellular citrate content was reduced to 50% by oleate (table 6).

With respect to the regulation of gluconeogenesis it is noteworthy that the cytosolic oxaloacetate concentration in the absence of exogenous substrate is around the K_M of phosphoenolpyruvate carboxykinase of 1–5 μM [32], but is increased 3-fold in the presence of lactate (or pyruvate), and about 9-fold in the presence of lactate plus oleate.

This may be related to the well-known stimulatory effect of long-chain fatty acids on gluconeogenesis which has been shown to occur also in isolated liver cells [33]. The subcellular distribution of malate and citrate is shown on tables 5 and 6, respectively. Lactate or pyruvate markedly elevated both these metabolites in the cytosol up to concentrations exceeding the K_M values of the malate and citrate translocators [34,35]. An even larger accumulation of these metabolites was caused by lactate in the presence of oleate, leading to an almost equal distribution of malate between both compartments (table 5). Differently, the m/c ratios for citrate were above 5 under all metabolic conditions tested, but not as high as reported by [36] (table 6). The lack of cytosolic citrate in the presence of oleate suggests, that inhibition of the citrate transporter by long-chain acyl-CoA as shown with isolated mitochondria [37] might occur in the intact cell. This would also explain the absence of acetyl-CoA in the cytosol under these conditions (table 2) as being due to unavailability of substrate for citrate cleavage enzyme.

As to the validity of the digitonin method it is of interest, that with respect to the compartmentation of malate, citrate, acetyl-CoA and CoASH comparable results have been obtained by a different approach [38]. Moreover the ratios for malate/oxaloacetate, citrate/malate and NAD/NADH as derived from our data are in agreement with those found by others using different procedures. Thus, the malate/oxaloacetate ratios of 30–130 as reported here are well in the range obtained in freeze-clamped liver [39]. Furthermore, in this study the citrate/malate ratio was found to be 4 times higher in the mitochondria than in the cytosol, while a gradient of about 3 against the incubation medium was maintained by isolated liver mitochondria [40]. If our cytosolic malate and oxaloacetate concentrations are used for the calculation of the cytosolic NAD/NADH ratio from the equilibrium constant for

Table 5
Subcellular distribution of malate in isolated hepatocytes from fasted rats in the presence of various substrates

Substrate added	mM	Amount (nmol/g dry wt.) Total	Pellet	(T – P)	Concentration (mM) Mit.	Cyt.	Concentration gradient (m/c)
None		113 ± 35 (6)	61 ± 22 (6)	52	0.38	0.034	11.2
Lactate	10	556 ± 104 (5)	102 ± 45 (5)	454	0.63	0.33	1.9
Oleate	0.9	94 ± 20 (5)	53 ± 17 (5)	41	0.33	0.026	12.7
Oleate + lactate	0.9 10	4108 ± 365 (9)	561 ± 80 (9)	3547	3.45	2.59	1.3
Pyruvate	7	485 ± 86 (6)	95 ± 20 (6)	390	0.58	0.28	2.1
Aspartate	7	129 ± 26 (6)	66 ± 17 (6)	63	0.40	0.043	9.3
Ethanol	12	112 ± 33 (6)	55 ± 10 (6)	57	0.34	0.043	7.9

malate dehydrogenase [28] the values in the presence of lactate or lactate plus oleate are 763 and 249, respectively. By calculation from the lactate/pyruvate couple values of 725 and 528 have been arrived at for the cytosolic NAD/NADH ratio in the freeze-clamped liver from fed and fasted rats, respectively [28]. The mitochondrial NAD/NADH ratio of hepatocytes supplied with lactate or both lactate and oleate as derived from the 3-hydroxybutyrate-acetoacetate system [28,41] was 120 and 21, respectively. In agreement with [36] this ratio is by a factor of about 10 higher, if calculated from the mitochondrial malate-oxaloacetate couple considering our values to represent the free concentrations. With respect to oxaloacetate

Table 6
Subcellular distribution of citrate in isolated hepatocytes from fasted rats in the presence of various substrates

Substrate added	mM	Amount (nmol/g dry wt.) Total	Pellet	(T – P)	Concentration (mM) Mit.	Cyt.	Concentration gradient (m/c)
None		260 ± 61 (5)	152 ± 34 (5)	108	0.94	0.079	11.9
Lactate	10	1676 ± 222 (5)	840 ± 222 (5)	836	5.14	0.61	8.4
Oleate	0.9	128 ± 44 (6)	137 ± 60 (6)	–	0.86	–	–
Oleate + lactate	0.9 10	4825 ± 300 (6)	1942 ± 189 (6)	2883	11.82	2.11	5.6
Pyruvate	7	1463 ± 42 (4)	773 ± 81 (4)	690	4.71	0.51	9.2
Ethanol	12	200 ± 14 (5)	152 ± 46 (5)	48	0.94	0.034	27.6

the normality of binding sites has been estimated to be about 20 times higher than its concentration in the matrix [42]. Thus, if one does not doubt on the malate dehydrogenase reaction being in equilibrium, the above discrepancy by a factor of 10 could indicate, that some 90% of total mitochondrial oxaloacetate is present in bound form, yielding a free matricial concentration of 1–3 μM. Alternatively, the difference between the two redox systems could be explained on the assumption, that 3-hydroxybutyrate dehydrogenase and malate dehydrogenase do not share a common NAD-pool [41].

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