INSULIN ACTIVATES PYRUVATE DEHYDROGENASE BY LOWERING THE MITOCHONDRIAL ACETYL-CoA/CoA RATIO AS EVIDENCED BY DIGITONIN FRACTIONATION OF ISOLATED FAT CELLS

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Received 10 July 1978

1. Introduction

For an advancement in metabolic regulation, knowledge of the subcellular distribution of metabolic intermediates appears of great importance. As an experimental approach for rapid separation of the cytosolic and the mitochondrial cellular compartments the digitonin procedure [1] has been successfully used for studies on the regulation of pyruvate dehydrogenase (PDH) interconversion [2,3], and on the mechanism of glucagon action [4] in isolated rat liver cells. No information is available so far on metabolite compartmentation in adipose tissue. In this study we describe a procedure using digitonin for the fractionation of isolated fat cells and present data on the distribution of ATP, ADP, CoA-SH and acetyl-CoA between the cytosolic and the mitochondrial compartments of rat epididymal fat cells. Furthermore, by this technique we could demonstrate that insulin leads to marked changes of the mitochondrial acetyl-CoA/CoA-SH ratio suggesting that regulation of PDH interconversion by insulin is achieved through control of the activity of PDH kinase.

2. Materials and methods

Enzymes, coenzymes and carbonylcyanide-*p*-trifluormethoxy-phenylhydrazone (CFCCP) were from Boehringer, Mannheim, bovine serum albumin (BSA), dried, purified, from Behring Werke AG, Marburg-Lahn, collagenase CLS from Worthington, Freehold, morpholinopropane sulfonic acid (MOPS) from Serva, Heidelberg, Lubrol PX from ICI, Frankfurt, silicon oil AK 20 from Wacker Chemie, Burghausen. Other chemicals were from Merck, Darmstadt. Pig insulin was a gift of Novo, Mainz.

ATP [5], ADP [6], and CoA-SH [7] were determined by enzymatic methods with an Aminco-Bowman spectrophotofluorometer (Am. Inst. Co.). Acetyl-CoA was measured isotopically by the formation of a stoichiometric amount of labelled citrate after enzymatic condensation with ^{14}C-labelled oxaloacetate [8]. PDH (EC 1.2.4.1) activity was measured spectrophotometrically before (PDH_a), and after incubation of the extracts with pig heart phosphatase [9] and 10 mM Mg^{2+} as in [10]. Glutamate dehydrogenase (GlDH) (EC 1.4.1.2) and lactate dehydrogenase (LDH) (EC 1.1.1.27) were assayed by standard methods [11].

Isolated fat cells were prepared from epididymal adipose tissue of normal fed Sprague-Dawley rats (W. Gassner, Sulzfeld; E. Jautz, Kisslegg) 160–200 g, by the collagenase method [12] using about 2 ml/g fat pad of a solution: 20 mM Tris–HCl; 97.5 mM NaCl; 5 mM K_2HPO_4 ('Tris-phosphate'); 0.5 mM glucose; 50 mg BSA/ml; and 260 U collagenase; final pH 7.4, for the digestion.

Cell incubations: 3.5 ml packed cells were incubated with gentle shaking in open plastic flasks at 37°C in 19 ml Tris-phosphate, pH 7.4, containing 5.5 mM glucose, 10 mM $NaHCO_3$, 25 mg BSA/ml, and the additions specified otherwise. After 20 min the cells were separated by centrifugation for 10 s at 400 × *g*, the aqueous infranatant drawn off, and the cells resuspended with 10 ml MOPS buffer (0.25 M sucrose,

20 mM MOPS, 3 mM EDTA, pH 7.4) at 37°C. The suspension was recentrifuged at 400 × *g* for 30 s at room temperature, and after removal of the infranatant the cells were equally dispersed by blowing through a stream of 95% O_2/5% CO_2 for 2 s. Cell fresh weight was determined after centrifuging 0.2 ml samples of packed cells through 0.5 ml dinonylphthalate for 3 s in an Eppendorf centrifuge model 3200.

For the separation of the soluble and particulate compartment 1 ml packed cells from the incubations was injected into a homogenizer cup (Mini-Potter, Braun, Melsungen) filled with 1.9 ml ice cold MOPS-buffer containing 1.9 mg digitonin, and immediately cooled down to 2–4°C by mixing with the teflon pestle dipped in liquid N_2 before use. After homogenisation (2 strokes in 10 s) with the motor-driven pestle two 1 ml portions were transferred in two Eppendorf cups, and centrifuged at 19 000 × *g* for 90 s in an Eppendorf centrifuge (model 3200) which was placed in a refrigerator and was cooled before with liquid N_2. The temperature after centrifugation was 2–4°C.

For determination of the cytosolic adenine nucleotides the aqueous phase of one cup was rapidly aspirated into a syringe containing $HClO_4$ to yield final conc. 6%. The aqueous phase of the other cup was sampled and frozen in liquid N_2 for enzyme determinations. Immediately after removal of the aqueous phases the cups were plunged into liquid N_2. The frozen pellets comprising the mitochondrial space were either deproteinized with 0.2 ml 6% HClO by vigorous agitation on a Whirl-Mix, or extracted for enzyme determinations as described below. To have time for these manipulations each incubation was started separately with a 5 min interval. The deproteinized samples were neutralized with KOH, centrifuged and the clear supernatants used for metabolite analyses. For acetyl-CoA determinations the extracts were neutralized with 2 M Tris as it appeared that some acetyl-CoA was lost upon centrifugation with the $KClO_4$ precipitate.

For determinations of total cellular metabolites packed fat cells (0.2 ml) not exposed to digitonin were directly mixed with $HClO_4$ washed once with diaethylether and neutralized as described.

Extraction for enzyme determinations: Fat cells and the mitochondrial pellets, respectively were homogenized at 0°C with a high speed tissue disintegrator (Ultra-Turrax TP 10, Jahnke und Kunkel, Stauffen) 2 times 20 s each with a 20 s interval. The medium was a mixture of 1 ml 5 mM Tris–HCl, pH 7.0 containing 0.05 ml of rat serum as stabilizer for PDH [13], 0.01 ml Lubrol PX, and 2.5 ml silicon oil. The homogenates were centrifuged for 1 min at 13 000 × *g* and the clear infranatants used for enzyme determinations.

In later experiments enzyme extracts were prepared as follows: The frozen cells or pellets after addition of the above mixture (at omission of silicon oil) were homogenized in the frozen state (liquid N_2) 20 s with a micro dismembrator (Braun, Melsungen). The frozen powder was transferred to an Eppendorf cup filled with 0.1 ml silicon oil AK 20, and centrifuged for 2 min at 15 000 × *g* at 5°C.

3. Results and discussion

The distribution of mitochondrial and cytosolic marker enzymes after digitonin fractionation of adipocytes is shown in table 1. The pellet fraction contained 85%, 82%, and 7% of the total cellular amount of PDH, GlDH, and LDH, respectively, indicating good separation of the two compartments has been achieved. In the cytosolic fraction PDH and GlDH activities were too low for being exactly quantitated so that their contribution to the overall balance cannot be firmly established. The possibility that some mitochondria might be burried in the fat cake was excluded as the recovery of GlDH remained the same (83%) when the pellet was extracted together with the fat plug. The LDH activity of the cytosolic fraction seems to exceed the total cellular activity. This may be due to some loss of LDH from leaky cells during centrifugation on dinonylphthalate, a step not applied for cells due to digitonin fractionation.

The distribution of ATP and ADP between the particulate and the soluble compartment of fat cells after 20 min incubation in the presence of glucose is shown in table 2. 7% and 12% total cellular ATP and ADP, respectively, was found in the mitochondrial fraction, and the remainder in the cytosol. This distribution results in mitochondrial and cytosolic ATP/ADP ratios of 2.1 and 4.9, respec-

Table 1
Distribution of marker enzymes between the pellet and the soluble fractions of isolated fat cells after digitonin treatment

	Whole cells	Pellet fraction	Soluble fraction
PDH_{total}	157.6 ± 8.5 (32)	134.2 ± 6.4 (31)	traces
GlDH	990 ± 28.7 (31)	815.2 ± 25.0 (30)	traces
LDH	10 749 ± 369 (29)	794 ± 40 (24)	12 739 ± 484 (31)

Enzyme activities ± SEM with the number of experiments in parentheses are given in mU/g fresh cells. Digitonin fractionation was carried out as indicated in section 2. The homogenates were centrifuged for 60 s. The fat plugs were removed before extracting the pellets. For further details see section 2

tively, not much different from that observed in isolated rat hepatocytes [1,2].

Earlier studies suggested that the effect of insulin on PDH activity to increase the active form of the enzyme might be explained by a lowering of the phosphorylation state of the mitochondrial adenine nucleotides [14]. The results of our compartmentation studies also shown in table 2 are not in support of this view. There was no change of the mitochondrial ATP/ADP ratio in fat cells incubated with a dose of insulin which caused an increase of the level of PDH_a by about 100%. Control experiments with an uncoupler of oxidative phosphorylation which leads to a similar increase of PDH_a as does insulin confirmed that a decrease of the mitochondrial ATP/ADP should have been well detectable with our method.

Table 2
Subcellular distribution of adenine nucleotides and PDH activities in isolated rat fat cells. Effect of insulin (1 mU/ml) and uncoupling of oxidative phosphorylation

Compartment	Additions	PDH_a	PDH_{total}	ATP	ADP	ATP/ADP
		(mU/g fresh cells)		(nmol/g fresh cells)		
Whole	none	63 ± 8	220 ± 26	130 ± 5	36 ± 4	4.3 ± 0.6
Cells	insulin	116 ± 12[c]	227 ± 28	145 ± 13	35 ± 5	4.9 ± 0.7
	CFCCP	112 ± 21[c]	209 ± 26	137 ± 12	41 ± 8	4.1 ± 0.7
Pellet	none	68 ± 12	226 ± 34	9.5 ± 0.7	4.5 ± 0.4	2.1 ± 0.1
Fraction	insulin	116 ± 16[c]	229 ± 34	8.7 ± 0.6[b]	4.2 ± 0.3	2.2 ± 0.2
	CFCCP	131 ± 32[c]	208 ± 38	5.2 ± 0.6[c]	8.3 ± 0.8[c]	0.7 ± 0.1[c]
Soluble	none			122 ± 4.4	31 ± 4	4.9 ± 0.8
Fraction[a]	insulin			137 ± 13	31 ± 4	5.3 ± 0.8
	CFCCP			132 ± 12	33 ± 8	6.3 ± 1.5

[a] Whole cells minus pellet fraction
[b] $P < 0.05$
[c] $P < 0.01$

Cells were incubated 20 min in glucose containing medium and further treated for digitonin fractionation as described in section 2. CFCCP was added in 20 µl methanol to give final conc. 2 µM. Methanol alone was ineffectual. Mean values ± SEM of 10 different experiments assayed in duplicate are given. Statistical significance against control without insulin was measured by Student's *t* test

Table 3
Effect of insulin (2 mU/ml) on the subcellular distribution of CoA-SH and acetyl-CoA in isolated fat cells

Compartment		CoA-SH	Acetyl-CoA	Acetyl-CoA
		(nmol/g fresh cells)		CoA-SH
Whole Cells	control	3.25 ± 0.82	1.58 ± 0.14	0.52 ± 0.13
	insulin	4.69 ± 1.01[a]	2.84 ± 0.62[a]	0.63 ± 0.16[a]
Pellet Fraction	control	2.21 ± 0.82	1.24 ± 0.53	0.69 ± 0.48
	insulin	3.32 ± 0.85[a]	0.59 ± 0.23[a]	0.20 ± 0.10[a]
Soluble Fraction	control	1.75 ± 1.22	0.70 ± 0.20	0.57 ± 0.27
	insulin	2.63 ± 1.78[a]	1.55 ± 0.24[a]	0.78 ± 0.36

[a] $P < 0.01$

Mean values ± SEM from 7 different experiments, each being assayed in triplicate are given. Mitochondrial PDH_a activity (mU/g fresh cells) was 62.1 ± 32.2 for the control and 106.6 ± 27.4[a] for samples incubated with insulin, total PDH activity (mU/g fresh cells) was 272.3 ± 63.8 and 293.2 ± 57.8, respectively. Statistical significance against control without insulin was measured by Student's *t* test

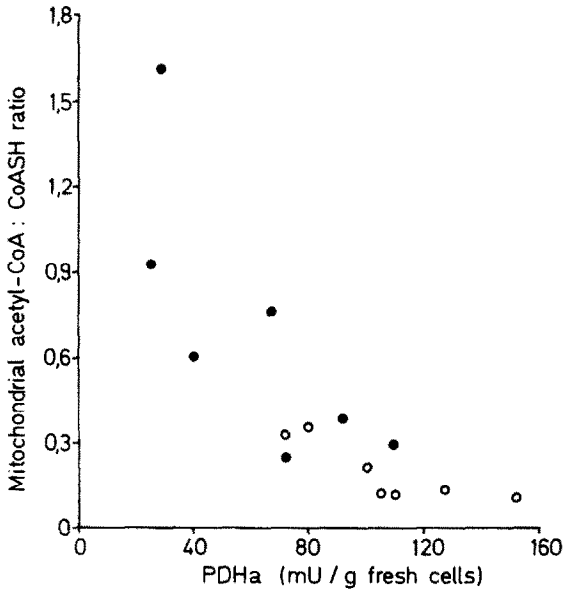

Fig.1. Correlation between the PDH_a-activity and the acetyl-CoA/CoA-SH ratio in the pellet fraction of digitonin fractionated adipocytes. For experimental details see section 2. (•) Control, (○) 2 mU insulin/ml. The symbols are the mean values of triplicate determinations of the 7 single experiments of table 3.

The subcellular distribution of CoA-SH and acetyl-CoA in fat cells is shown in table 3. About 70% and 80% total CoA-SH and acetyl-CoA, respectively, of glucose incubated cells is located in the mitochondrial space. This distribution pattern is strikingly changed upon incubation with insulin which leads to a reduction of the acetyl-CoA inside the mitochondria compensated by an increase in the cytosol. Thereby the mitochondrial acetyl-CoA/CoA-SH ratio drops from 0.69 to 0.20, an effect which is masked if one calculates the acetyl-CoA/CoA-SH ratio from the total cellular metabolite contents. In agreement with observations on fat pads [15] there was also a significant increase of the CoA-SH and acetyl-CoA content of whole fat cells after insulin. Compartmentation reveals that the elevation of CoA-SH takes place in the cytosol and intramitochondrially, and that of acetyl-CoA only in the cytosol.

Studies on purified preparations of the bovine kidney and heart pyruvate dehydrogenase complexes have shown that acetyl-CoA and NADH stimulate the activity of PDH_a kinase, whereas CoA-SH and NAD^+ inhibit kinase activity [16]. Accordingly, the steady state activity of the PDH complex is lowered at increasing molar ratios of acetyl-CoA/CoA-SH

and/or NADH/NAD^+ [16]. In fig.1 we have plotted the mean values of the mitochondrial acetyl-CoA/CoA-SH ratios from the experiments indicated in table 3 against the levels of PDH_a which were measured in parallel in the same pellets. There is a clear correlation from which it can be derived that a 10-fold decrease of the acetyl-CoA/CoA-SH ratio is accompanied by a 5-fold increase of the level of PDH_a.

Our studies clearly demonstrate that insulin, while not altering the phosphorylation state of the adenine nucleotides lowers the ratio of acetyl-CoA/CoA-SH in the mitochondrial compartment of fat cells, the site where the PDH complex is located. On the reasonable assumption that the enzyme of adipose tissue has similar regulatory properties as that of kidney or heart muscle our results offer strong evidence for the participation of the mitochondrial acetyl-CoA/CoA-SH couple in the mechanism by which insulin controls the phosphorylation state of the PDH complex in adipose tissue. Whether additional factors such as the redox state of the mitochondrial pyridine nucleotides, or others are involved in this control must remain open.

Acknowledgements

The skillful technical assistance of Miss Monica Wirtz is greatly acknowledged. We wish to thank Dr E. A. Siess for valuable suggestions and discussions during this work. This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, FRG.

References

[1] Zuurendonk, P. F. and Tager, J. M. (1974) Biochim. Biophys. Acta 333, 393–399.
[2] Siess, E. A. and Wieland, O. H. (1975) FEBS Lett. 52, 226–230.
[3] Siess, E. A. and Wieland, O. H. (1976) Biochem. J. 156, 91–102.
[4] Siess, E. A., Brocks, D. G., Lattke, H. and Wieland, O. H. (1977) Biochem. J. 166, 225–235.
[5] Lamprecht, W. and Trautschold, I. (1970) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U. ed) vol. 2, p. 2024, Verlag Chemie, Weinheim/Bergstraße.
[6] Jaworek, D., Gruber, W. and Bergmeyer, H. U. (1970) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U. ed) vol. 2, p. 2051, Verlag Chemie, Weinheim/Bergstraße.
[7] Garland, P. B. (1974) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U. ed) vol. 2, pp. 2029–2035, Verlag Chemie, Weinheim/Bergstraße.
[8] Rössle, M. (1975) Dissertation, Medizinische Fakultät der Universität Freiburg.
[9] Siess, E. A. and Wieland, O. H. (1972) Eur. J. Biochem. 26, 96–105.
[10] Portenhauser, R. and Wieland, O. H. (1972) Eur. J. Biochem. 31, 308–314.
[11] Bergmeyer, H. U., Gawehn, K. and Grassl, M. (1970) in: Methoden der enzymatischen Analyse (Bergmeyer, H. U. ed) vol. 1, pp. 420, 442, Verlag Chemie, Weinheim/Bergstraße.
[12] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[13] Wieland, O. H. (1975) FEBS Lett. 52, 44–47.
[14] Wieland, O. H., Weiss, L., Löffler, G., Brunner, I. and Bard, S. (1975) in: Metabolic Interconversion of Enzymes (Shaltiel, S. ed) p. 125, Springer-Verlag, Berlin, Heidelberg, New York.
[15] Denton, R. M. and Halperin, M. L. (1968) Biochem. J. 110, 27–38.
[16] Pettit, F. H., Pelley, J. W. and Reed, L. J. (1975) Biochem. Biophys. Res. Commun. 65, 575–582.