REGULATION OF PYRUVATE DEHYDROGENASE INTERCONVERSION IN ISOLATED HEPATOCYTES BY THE MITOCHONDRIAL ATP/ADP RATIO

E. A. SIESS and O. H. WIELAND

Forschergruppe Diabetes und Klinisch-chemisches Institut des Krankenhauses München-Schwabing, 8 München 40, Kölner Platz 1 West Germany

Received 3 February 1975

1. Introduction

Recently Wieland and Portenhauser have shown that the degree of pyruvate dehydrogenase (PDH) (EC 1.2.4.1.) phosphorylation in isolated rat liver mitochondria is related to the phosphorylation state of intramitochondrial adenine nucleotides (AN) [1]. In order to see whether this mechanism is operative also in the intact cell we studied the effect of inhibitors of ATP generation or translocation on PDH-activities and mitochondrial AN levels in isolated hepatocytes. By taking advantage of the method developed by Zuurendonk and Tager [2] for the separation of mitochondrial and extramitochondrial compartments we could demonstrate that, like in isolated mitochondria, a clear correlation between PDH phosphorylation and the mitochondrial ATP/ADP ratio exists in intact liver cells.

2. Materials and methods

Enzymes, coenzymes and carboxyatractyloside were purchased from Boehringer (Mannheim, Germany). Carbonylcyanide-*m*-chlorophenyl-hydrazone (CCCP) was obtained from Sigma (St. Louis, USA). Glucose, ethanol, digitonin were products of Merck (Darmstadt, Germany). Digitonin was used after recrystallization from hot ethanol.Collagenase was purchased from Worthington (Freehold, USA). Gelatin 'non plus ultra' grade and morpholinopropane sulfonic acid (MOPS) came from Serva (Heidelberg, Germany). Silicon oils AR 200 (No. I/9618) and AR 20 (No. I/9674) were obtained from Wacker-Chemie (Burghausen, Germany). Lubrol PX was supplied by ICI (Frankfurt, Germany).

Isolated liver cells from normal fed male Sprague Dawley rats were prepared essentially according to [3] except that calcium-free Krebs-Henseleit bicarbonate buffer containing 1.5% gelatin (KH-G) without substrates was used, and that the cells were washed twice only. About 90% of the cells excluded 0.2% trypane blue. The incubation mixture consisted of 0.55 ml KH-G, pH 7.4, 0.01 ml 80 mM $CaCl_2$ and 0.2 ml of liver cell suspension in KH-G corresponding to about 70–80 mg fresh liver. Incubation was performed at 37°C in stoppered plastic tubes (10 × 1.9 cm) with 95% O_2 – 5% CO_2 as the gas phase in a shaking water bath for 5 min. Separation of soluble ('extramitochondrial') and insoluble ('mitochondrial') cell constituents was achieved at 0 – 4°C as follows: for AN determination, 0.1 ml of the incubation mixture was injected into 1 ml of an ice-cold solution of 0.25 M sucrose, 20 mM MOPS buffer, pH 7.0, 3 mM EDTA and 4 mg digitonin (digitonin-medium). The temperature after mixing was 2.4 ± 0.2°C as determined with a thermoelement (Tastotherm P 60, Deutsche Gulton GmbH, Frankfurt, Germany). This mixture was shaken in an Eppendorf 'Rotationsmischer' type 3300 for 15 sec, then 1.0 ml was transferred to an Eppendorf Cup containing 0.12 ml of 12% perchloric acid (PCA) beneath a layer of 0.5 ml of a silicon oil mixture (AR 200: AR 20 = 1:1) which was then placed in the Eppendorf centrifuge (type 3200) rotor. Centrifugation for 10 sec was started 30 sec after mixing with digitonin. For AN measurements of the 'soluble' compartment, 0.8 ml of the supernatant were rapidly mixed with 0.2 ml of 70% PCA and neutralized with 10 N KOH shortly before assay.

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For enzyme measurements 0.1 ml of the incubation mixture was mixed with digitonin-medium in parallel by a second person, and shaken as above. After centrifugation for 10 sec the supernatant was rapidly removed and the pellet quickly frozen in liquid N_2. To have sufficient time for these manipulations incubations were started every two min by the addition of liver cells.

For AN measurements of the 'particulate' compartment the oil phase was carefully sucked off and the pellet was suspended in the PCA phase after further addition of 0.2 ml 12% PCA. After a second extraction with 0.2 ml 12% PCA the combined supernatants were neutralized as above.

ATP and ADP were determined by enzymatic methods [4] using an Eppendorf photometer and a Perkin Elmer dual wavelength spectrophotometer, respectively. Results are expressed as μmol per g wet weight on the basis of 10^8 cells corresponding to 1 g wet liver [3].

For PDH extraction the pellets were suspended in 0.3 ml of 20 mM potassium phosphate buffer pH 7.0, containing 50 μl/ml rat serum as stabilizer [5] and 10 μl/ml Lubrol PX and shaken for 5 min at 2–4°C. The supernatant after centrifugation was collected, the pellet frozen again in liquid N_2 and reextracted with 0.2 ml buffer as described. The combined extracts containing at least 95% of the PDH-activity were assayed before (= PDH_a) and after complete activation with PDH-phosphatase [6] (= total PDH) essentially as described [7]. When kept on ice no change in the active from or in total PDH activity occurred within 60 min.

Digitonin in the concentration routinely used (even up to 6 mg/ml) did not affect PDH activity. Lactate dehydrogenase (LDH) (EC 1.1.1.27), myokinase (MK) (EC 2.7.4.3) and glutamate dehydrogenase (GDH) (EC 1.4.1.2) were assayed by standard methods [4].

3. Results and discussion

3.1. *Adenine nucleotides in the soluble and insoluble fractions of hepatocytes*

The release of LDH, MK, and GDH as marker enzymes for cytosol, intermembrane space and mitochondrial matrix, respectively, is shown in table 1 to be a function of the incubation time and the digitonin concentration applied to the hepatocytes. As may be seen, the release of LDH was highly dependent on digitonin concentration and incubation time whereas that of GDH was almost independent from both parameters. Under our standard conditions mean values for the leakage of LDH, MK, and GDH were 80.7 ± 0.7% (n = 76), 29.0 ± 1.4% (n = 50), and 2.9 ± 0.2% (n = 69), respectively. The low release of GDH indicates that the inner mitochondrial membrane was practically unaffected by digitonin. The leakage of MK from the intermembrane space is of no bearing on our problem, since the outer membrane is not essential for the compartmentation of AN.

Direct evidence that digitonin caused no significant leakage of AN from the pellet fraction is provided by the data presented in table 2. Although 2 mg/ml digitonin was sufficient to obtain a constant ATP/ADP

Table 1
Release of marker enzymes from isolated hepatocytes exposed for different times to increasing concentrations of digitonin

Digitonin (mg/ml)	Time of incubation								
	20 sec			30 sec			40 sec		
	LDH	MK	GDH	LDH	MK	GDH	LDH	MK	GDH
0	3.3	1.2	0.5	4.4	1.5	0.9	7.2	1.1	0.9
1	17.9	5.8	3.2	36.0	6.0	2.7	69.4	5.7	1.7
2	24.3	4.9	2.6	64.0	6.0	2.2	77.4	8.9	2.7
4	34.8	7.8	2.6	72.1	12.0	2.7	80.0	19.8	3.1

Values indicate enzyme activities in the supernatant fraction and are expressed in percent of the sum found in the supernatant and the pellet fractions.

Table 2
ATP and ADP levels (μmol/g fresh weight) in the pellet fraction of isolated rat liver cells as a function of digitonin concentration and incubation time.

Digitonin (mg/ml)	Time of incubation					
	20 sec		30 sec		40 sec	
	ATP	ADP	ATP	ADP	ATP	ADP
0	1.98	0.68	2.06	0.71	2.06	0.58
1	0.71	0.44	0.57	0.33	0.53	0.28
2	0.68	0.33	0.46	0.32	0.55	0.28
4	0.57	0.24	0.49	0.28	0.51	0.27

Without digitonin no AN were found in the supernatant fraction. The amount of nucleotides released from the pellet by digitonin was fully recovered in the soluble fraction.

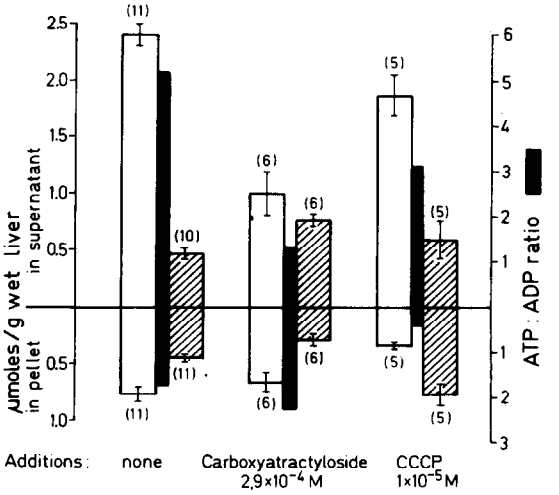

Fig.1. Compartmentation of ATP (□) and ADP (▨) in isolated liver cells from fed rats. For experimental details see Materials and methods. Carboxyatractyloside was dissolved in KH-G and added in 0.05 ml to give the final concentration indicated. The uncoupler was added as described in the legend to table 3.

ratio, incubations were routinely performed at 4 mg/ml digitonin for 30 sec because of better enzyme separation (see table 1).

According to the AN distribution pattern in fig.1, 75% of the ATP were found in the extramitochondrial

Table 3
Effect of uncoupling of oxidative phosphorylation by CCCP on AN in the soluble and insoluble compartments of isolated rat liver cells.

[CCCP] μM	Supernatant			Pellet		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
0	2.40 ± 0.11 (11)	0.47 ± 0.05 (10)	5.11	0.76 ± 0.06 (11)	0.45 ± 0.03 (11)	1.69
5	2.38 ± 0.20 (5)	0.62 ± 0.22 (5)	3.84	0.68 ± 0.07 (5)	0.56 ± 0.10 (5)	1.21
6.6	2.18 ± 0.37 (3)	0.59 ± 0.20 (3)	3.69	0.55 ± 0.07 (3)	0.62 ± 0.01 (3)	0.89
10	1.86 ± 0.19 (5)	0.60 ± 0.17 (5)	3.10	0.34 ± 0.03 (5)	0.77 ± 0.10 (5)	0.44
13.3	1.23 ± 0.44 (3)	0.65 ± 0.13 (3)	1.89	0.18 ± 0.02 (3)	0.77 ± 0.12 (3)	0.23
20	0.86 ± 0.32 (4)	0.69 ± 0.10 (5)	1.25	0.21 ± 0.01 (4)	0.82 ± 0.08 (5)	0.26

Experimental details as described in Materials and methods except that CCCP in 5 μl methanol was added to the incubation medium to yield the final concentrations indicated. Methanol alone was ineffectual. Mean values ± S.E.M. are given, number of experiments in parentheses.

and 25% in the 'mitochondrial' compartment. ADP was equally distributed between the two spaces. The resulting ATP/ADP ratios of 5.2 and 1.7, respectively confirm the results of Zuurendonk and Tager [2], and are similar to those of Elbers et al. [10] obtained by a different approach. Total ATP and ADP levels of 3.16 and 0.92 μmol/g fresh liver, respectively, agree well with those found by Krebs et al. [8] in unfractionated isolated liver cells, but are higher than the values of others [2,9]. Furthermore fig.1 demonstrates the changes in the ATP and ADP levels that occur when atractyloside or an uncoupler such as CCCP was added to the incubation medium. Again in agreement with

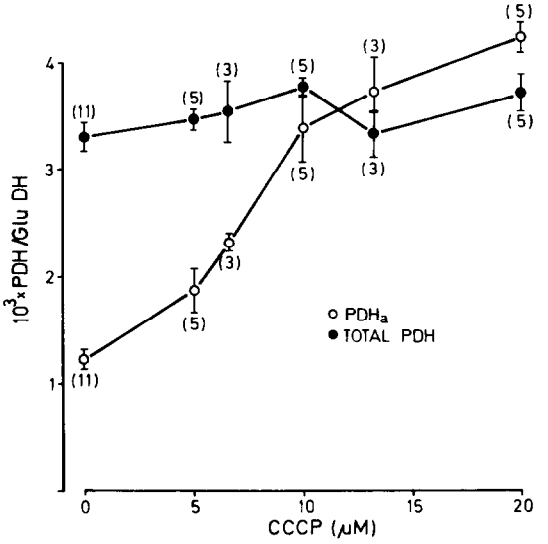

Fig.2. Effect of CCCP on the state of the PDH system in isolated liver cells from fed rats. Experimental details are those described in the legend to table 3. Mean values ± S. E. M. are given, number of experiments in parentheses.

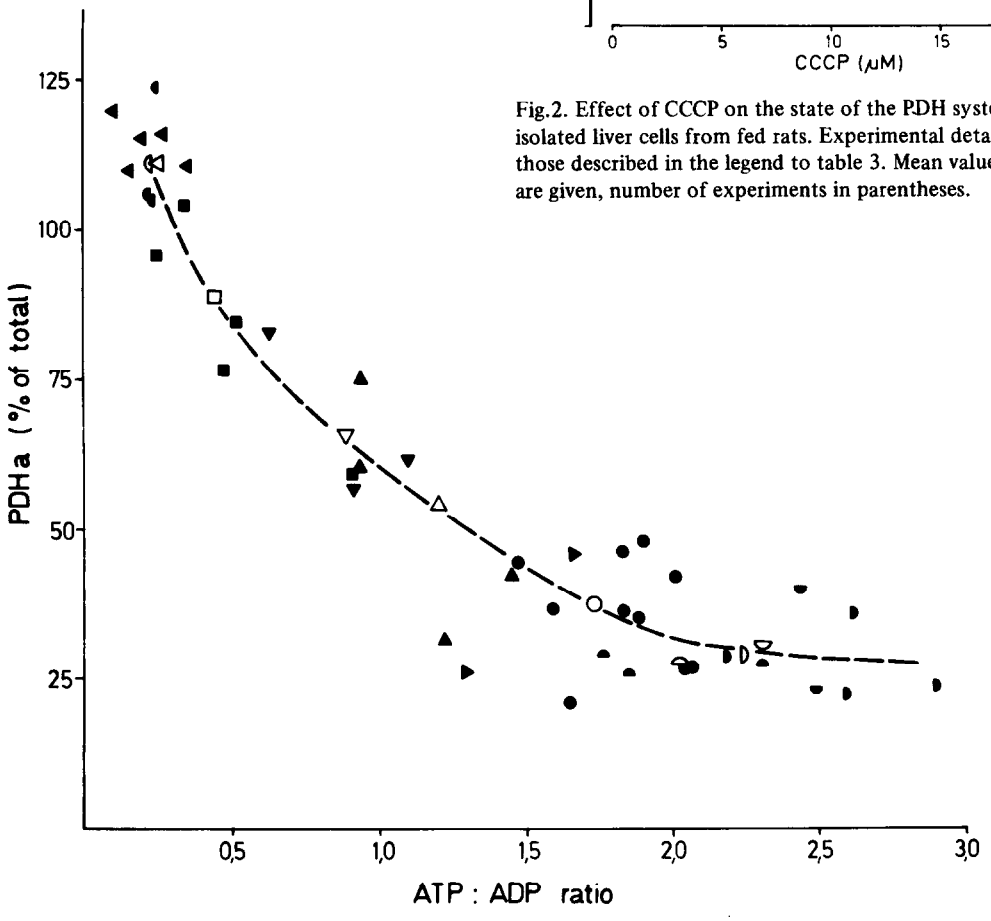

Fig.3. Correlation between the state of the PDH system and the ATP/ADP ratio in the pellet fraction of digitonin treated isolated rat liver cells. For experimental details see Materials and methods. (•) Control. Final concentrations of CCCP: (▶) 2.5 μM; (▲) 5 μM; (▼) 6.6 μM; (■) 10 μM; (◀) 13.3 μM; (◀) 20 μM. Final concentrations of carboxyatractyloside: (◢) 0.0725 mM; (◥) 0.145 mM; (◗) 0.29 mM. The open symbols are the mean values of the single experiments the latter represented by the corresponding filled symbols.

[2] we observed a marked decrease of the ATP/ADP ratio by atractyloside in the cytosol and an increase in the 'mitochondrial' compartment, due to a lowering of ADP. CCCP caused a marked fall of the ATP/ADP ratio in both compartments by reducing the ATP and increasing the ADP level. The striking drop of extramitochondrial ATP caused by atractyloside could indicate that this compound, besides its known action on mitochondrial AN translocation, might also interfere with glycolytic ATP-supply.

Table 3 summarizes the changes of the ATP and ADP levels in the supernatant and the pellet fractions at various concentrations of CCCP.

3.2. *PDH phosphorylation at various ATP/ADP ratios*

It is well established that ADP inhibits PDH-kinase competitively to ATP [11–14]. Furthermore, studies with isolated mitochondria [1] have shown that PDH interconversion is related to the phosphorylation state of the mitochondrial AN. That this mechanism operates also in intact cells is evidenced by the data illustrated in fig.2. As may be seen, the dose-dependent lowering of the ATP/ADP ratio caused by CCCP (see table 3) was paralleled by a gradual increase of PDH_a without significant changes of total PDH. When the ATP/ADP ratios of the pellet fractions from isolated liver cells incubated under different conditions are plotted against PDH_a an inverse relationship between PDH activity and the phosphorylation state of the AN is apparent (fig.3).

Present experiments indicate that regulation of PDH-interconversion by changes of the mitochondrial energy state in isolated hepatocytes is demonstrable not only with metabolic inhibitors but also by the application of different physiologically occurring substrates [15].

Acknowledgements

The skillful technical assistance of Mrs E. Gundel and Miss A. Schnabl during part of this study is greatly acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, Germany.

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