CELLULAR DISTRIBUTION OF PYRUVATE DEHYDROGENASE PHOSPHATASE. ACTIVITY IN NORMAL, HYPERINSULINIZED AND DIABETIC RATS

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1. Introduction

There are several reports in the literature suggesting a role of insulin in the regulation of the interconversion of pyruvate dehydrogenase (EC 1.2.4.1) between its active and inactive forms. In adipose tissue this hormone markedly increased the active dephospho form (PDHa) when applied in vivo or in vitro [1–4]. Also in liver a doubling of PDHa was observed shortly after insulin injection into normal rats [5]. Furthermore, PDH dephosphorylation in rat heart muscle was impaired in alloxan diabetes [6]. Stimulation of PDH-phosphatase by insulin in adipose tissue has been described [4,7], but was not confirmed by others [8]. Therefore we present our results on the activity of this enzyme in freeze-clamped tissues from normal, hyperinsulinized and alloxan diabetic rats. Some of these data have been reported elsewhere [6].

2. Materials and methods

Male Sprague-Dawley rats of 150–250 g (W. Gassner, Sulzfeld, Germany) were used. Induction of alloxan diabetes and insulin substitution were performed as described [9]. Preparation of tissue extracts: Organs were removed from rats under ether anaesthesia and frozen immediately with the aid of a Wollenberger clamp pre-cooled in liquid N_2. The rats were then decapitated and the heads kept in liquid N_2 until removal of the deep frozen brain. The organs were powdered in a Minidismembrator (Braun, Melsungen, Germany) driven at full speed for 30 sec and kept in liquid N_2 until extraction. For PDH-phosphatase extraction 1 g of powdered brain, kidney or liver was suspended in 3 ml, 1 g of heart muscle in 4 ml of 20 mM potassium phosphate buffer, pH 7.0, containing 1% Lubrol (ICI, Frankfurt, Germany) and 5% serum from fed Wistar rats [10] (medium A). Extraction from adipose tissue was performed by suspending 160 mg tissue powder with 0.4 ml of medium A and 2.4 ml silicon oil AK 20 (Wacker, Burghausen, Germany). The suspensions were thawed and homogenized in a high-speed tissue disintegrator (Ultra Turrax TP 18/2 Jahnke & Kunkel, Staufen i. Br., Germany) for 2 × 15 sec. The temperature did not exeed + 2°C. The homogenates were frozen, thawed and centrifuged for 2 min in an Eppendorf centrifuge (type 3200). The clear extracts were used for the enzyme assay. PDH-phosphatase assay by PDH reactivation: the reaction mixture consisting of 10 μl phospho-PDH (corresponding to 126 mU dephospho-PDH) 5 μl of a 1:1 mixture of 100 mM $MgCl_2$ and 5 mM $CaCl_2$, 5 μl of medium A and 5 μl of tissue extract was incubated for 20 min at 25°C and then analyzed for PDH activity spectrophotometrically as described [11] using lactate as the substrate. The tissue extracts were diluted with medium A to ensure linearity of PDH reactivation with protein and time up to 40 min. Blanks were run without phospho-PDH to measure the endogenous PDH activity of the tissue extracts and without tissue extract to detect traces of PDH-phosphatase activity in the phospho-PDH preparation. The values of PDH-phosphatase activity reported are appropriately corrected.

PDH-phosphatase assay by release of $^{32}P_i$ from [^{32}P]phospho-PDH: the reaction mixture consisted

of 20 μl [^{32}P]phospho-PDH (corresponding to about 150 μg protein containing 1 nmol $^{32}P_i$, corresponding to about 0.6 U dephospho-PDH), 5 μl 70 mM $MgCl_2$ or H_2O, 5 μl 20 mM potassium phosphate buffer, pH 7.0, and 5 μl of tissue extract. After incubation for 5 min at 25°C 10 μl of the reaction mixture were mixed with 20 μl 6% $HClO_4$ (w/v). After centrifugation the radioactivity in the supernatant was determined in a Packard liquid scintillation counter. The $^{32}P_i$ release due to Mg^{2+} was taken as a measure of phosphatase activity. (The addition of Ca^{2+} (up to 0.7 mM) or rat serum to the incubation mixture did not change the results).

Glutamate dehydrogenase (GDH) (EC 1.4.1.2), citrate synthase (CS) (EC 4.1.3.7), lactate dehydrogenase (LDH) (EC 1.1.1.27) and pyruvate kinase (PK) (EC 2.7.1.40) were assayed by standard methods [12]. PDH activity of tissue fractions was measured according to [5].

Protein was determined by the biuret method [17] employing KCN to correct for turbidity [18].

3. Results and discussion

The PDH-phosphatase activity in organs from normal, hyperinsulinized and alloxan-diabetic rats has been determined. The values found in kidney, heart muscle, liver, brain and epididymal adipose tissue are summarized in table 1. Per gram fresh weight or unit of glutamate dehydrogenase activity (not shown) neither hyperinsulinism nor insulin deficiency caused a statistically significant effect on PDH-phosphatase activity of any of the tissue extracts examined by its potency to reactivate exogenous phospho-PDH. Also no difference between normal and diabetic rats was found by following the release of $^{32}P_i$ from [^{32}P]phospho PDH (table 2). The validity of the latter method for PDH-phosphatase measurement however became dubious by the results obtained when the subcellular distribution of PDH-phosphatase in liver was studied with both methods. When tested by phospho-PDH reactivation the bulk of the phosphatase activity of a liver homogenate was recovered in the mitochondrial fraction together with glutamate dehydrogenase, citrate synthase and PDH (table 3). A completely different enzyme distribution pattern was obtained when the phosphatase measurement was attempted by [^{32}P]phospho-PDH dephosphorylation (fig.1). Only 18.9 ± 3.5% (n = 3) of total [^{32}P] phospho-PDH dephosphorylating activity of a liver homogenate was found in the mitochondrial fraction, 69.4 ± 3.6% (n = 3) being in the cytosol.

Thus it appears that most of the dephosphorylating activity of a liver homogenate is not related to phospho PDH reactivation. True PDH-phosphatase activity of

Table 1
Activity of pyruvate dehydrogenase phosphatase in tissue extracts from normal, hyperinsulinized and diabetic rats

Blood glucose (mg/100 ml)	Experimental group	Kidney	Heart muscle	Liver	Adipose tissue	Brain
117 ± 2 (9)	Control	391 ± 15.4 (6)	413 ± 73.1 (5)	170 ± 15 (9)	40.0 ± 4.5 (9)	480 ± 24.9 (6)
68 ± 6 (9)	Hyperinsulinized[a]	379 ± 20.9 (5)	456 ± 64.1 (6)	162 ± 16 (9)	39.7 ± 5.0 (9)	414 ± 31.5 (6)
44 ± 6 (4)	Alloxan-diabetic (insulin-treated)	360 ± 29.6 (4)	477 ± 76.3 (4)	222 ± 24.4 (4)	59.8 ± 11.3 (4)	530 ± 39.0 (4)
422 ± 32 (5)	Alloxan-diabetic (insulin withdrawn for 36 h)	421 ± 43.7 (5)	593 ± 75.1 (5)	221 ± 26.6 (5)	54.6 ± 10.3 (5)	438 ± 28.5 (4)

For details see Materials and methods. The results, expressed as mU/g fresh wt., are given as mean ± s.e.m. for the number of animals given in parentheses. The unit of phosphatase activity is defined by reactivation of one unit of phospho-PDH per min at 25°C.

[a]Animals received 10 U Altinsulin Hoechst i.p. 30 min before sacrifice, the control group was injected with saline.

Table 2
Release of $^{32}P_i$ from pig heart [^{32}P]phospho-PDH by tissue extracts from normal and alloxan-diabetic rats

Experimental group	Kidney		Heart muscle		Liver		Adipose tissue	
	A	B	A	B	A	B	A	B
Control	54.1 ± 3.7	0.89 ± 0.06 (10)	88.7 ± 6.9	2.00 ± 0.25 (10)	40.4 ± 2.8	0.37 ± 0.03 (10)	14.2 ± 1.6	1.98 ± 0.16 (8)
Alloxan-diabetic (insulin withdrawn for 36 h)	70.4 ± 8.7	0.85 ± 0.08 (12)	93.9 ± 11.1	2.13 ± 0.20 (12)	49.4 ± 3.8	0.37 ± 0.03 (12)	21.9 ± 3.2	1.61 ± 0.21 (12)

Phosphorylation of PDH was achieved as described in [14]. Some 93% and 99% of the radioactivity was released from the [^{32}P]phospho-PDH preparations by incubation for 15 and 90 min respectively with purified [14] PDH-phosphatase. The recovery of pig heart phosphatase added to the tissue extracts varied between 93% and 106%. For further details see Materials and methods. (Mean values ± s.e.m. are given for the numbers of animals in parentheses).

A = nmol $^{32}P_i$ released/g fresh wt. × 5 min.
B = nmol $^{32}P_i$ released/mg protein × 5 min.

Table 3
Intracellular distribution of PDH-phosphatase in normal rat liver as related to glutamate dehydrogenase, citrate synthase, pyruvate dehydrogenase, lactate dehydrogenase and pyruvate kinase

	Glutamate dehydrogenase (U/g fresh wt.)	%	Citrate synthase (U/g fresh wt.)	%	Pyruvate dehydrogenase (mU/g fresh wt.)	%	PDH-phosphatase (mU/g fresh wt.)	%	Lactate dehydrogenase (U/g fresh wt.)	%	Pyruvate kinase (U/g fresh wt.)	%
Homogenate	283 ± 18	100	6.0 ± 0.3	100	496 ± 99	100	111.2 ± 6.5	100	395 ± 71	100	20.3 ± 4.5	100
Supernatant (S)	13.6 ± 0.9	4.8	0.67 ± 0.02	11.2	20.7 ± 2.9	4.2	13.1 ± 1.5	11.8	321 ± 74	81.3	19.2 ± 2.7	94.6
Pellet (P)	230 ± 13	81.3	4.6 ± 0.5	76.7	437 ± 68	88.1	74.0 ± 5.6	66.5	100 ± 21	25.3	3.4 ± 0.3	16.7
Recovery in S + P		86.1		87.9		92.3		78.3		106.6		111.3

2 g of liver were suspended in 8 ml of 0.25 M sucrose in 20 mM potassium phosphate buffer, pH 7.0, and disrupted in a motor-driven Teflon–glass homogenizer at 4°C. The homogenate was separated into supernatant and pellet fractions by centrifugation at 10 000 *g* for 10 min. The pellet was frozen and extracted with medium A. Aliquots of the homogenate and the supernatant were kept in liquid N_2 and diluted 1:2 with medium A prior to use. (Mean values ± s.e.m. of three experiments are given). PDH-phosphatase was tested by the reactivation assay.

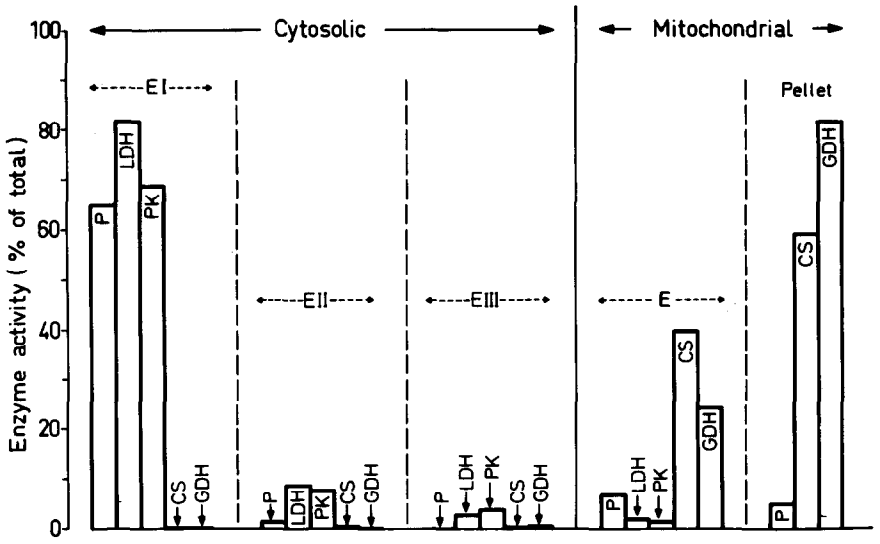

Fig.1. Release of $^{32}P_i$ from pig heart [^{32}P]phospho-PDH by the cytosolic and mitochondrial fractions of a normal fed rat liver. The cytosolic extracts (EI, EII, EIII) and the mitochondrial extract (E) were prepared according to Pette [15] except that 0.3 M sucrose in 20 mM potassium phosphate buffer, pH 7.0, was used. Of the radioactivity released by the supernatant or the mitochondrial fraction 93% and 102% respectively was eluted from Dowex 1 [16] by 2 N formic acid together with reference P_i. (P) $^{32}P_i$ releasing activity; (LDH) lactate dehydrogenase; (PK) pyruvate kinase; (CS) citrate synthase; (GDH) glutamate dehydrogenase.

tissue extracts should therefore be assayed rather by PDH reactivation than by $^{32}P_i$ release from labeled PDH. Whether [^{32}P]phospho-PDH dephosphorylation without reactivation occurs also in other tissues than liver has not been studied so far. Stansbie et al. [13], however, have claimed that in epididymal adipose tissue 80% of the PDH-phosphatase activity is localized in the extramitochondrial space. Since this observation was made by only following $^{32}P_i$ release from exogenous [^{32}P]phospho-PDH the question arises from our results with liver whether the cytosolic fraction of adipose tissue in fact contains PDH-phosphatase, i.e. an enzyme capable of reactivating phospho-PDH. According to Reed et al., PDH contains two seryl groups which are phosphorylated by PDH-kinase [19]. The one which is rapidly phosphorylated is essential for catalytic activity, while the second, reacting more slowly, does not correlate with enzyme activity. Thus the possibility exists that $^{32}P_i$ released from [^{32}P]phospho-PDH by the cytosolic fraction of liver and possibly other tissues stems from seryl residues not involved in PDH inactivation.

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