The Effect of Insulin on Pyruvate Dehydrogenase Interconversion in Heart Muscle of Alloxan-Diabetic Rats

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Summary. Evidence is presented for regulation by insulin of pyruvate dehydrogenase (PDH) interconversion in rat heart muscle in vivo and in vitro. In the alloxan diabetic rat the active (dephospho) enzyme amounted only to 12% of total PDH and was restored to 42% by insulin. Antilipolytic treatment of the diabetic animals was ineffective, indicating that the action of insulin was independent of a lowering of plasma non-esterified fatty acid concentration. On perfusion of isolated hearts from diabetic rats in the presence of glucose the proportion of pyruvate dehydrogenase in the active form remained low but was fully restored upon addition of insulin (2 mU/ml) to the medium. No effect of insulin was obtained in the absence of glucose. The correlation between the rate of pyruvate decarboxylation in the perfused heart and of pyruvate dehydrogenase activity, in vitro, suggests that in the diabetic heart the entry of pyruvate into the citric acid cycle is largely controlled by covalent modification of the pyruvate dehydrogenase complex rather than by feedback inhibition. The possible role of insulin therein is discussed.

Key words: Insulin, pyruvate dehydrogenase, enzyme interconversion, heart muscle, alloxan-diabetes, heart perfusion.

Inhibition of pyruvate oxidation is a well known alteration of heart muscle metabolism in diabetes and starvation [1]. The responsible enzyme, the pyruvate dehydrogenase (PDH, EC 1.2.4.1) complex is regulated by either feedback control by acetyl-CoA and NADH [1, 2] or by enzymatic phosphorylation and dephosphorylation reactions leading to the inactive form (PDH_b) or the active form (PDH_a), respectively [3, 4]. Previous work from this laboratory had shown that in heart muscle of normal fed rats about two-thirds of total PDH activity was found as PDH_a whereas only about one tenth of total activity was found as PDH_a in starved or ketoacidotic alloxan diabetic animals. Furthermore, PDH_a levels lowered by diabetes and by fasting could be normalized by insulin treatment and by refeeding of carbohydrate, or treatment with nicotinic acid, respectively [5]. From these and other experiments [6, 7] it was suggested that the release and oxidation of free fatty acids (FFA) might be an important factor for the regulation of PDH. The aim of the present work was to study the possible role of insulin in PDH interconversion in the diabetic heart muscle, in vivo and in vitro. Furthermore, tracer experiments were carried out in order to compare the rate of pyruvate oxidation in the perfused heart with the activity of PDH measured after extraction of the heart muscle. Preliminary reports have appeared elsewhere [8, 9].

Materials and Methods

Materials

Enzymes and coenzymes were products of Boehringer, Mannheim (Germany). 2-Deoxyglucose was from EGA-Chemie, Steinheim (Germany). Glucagon-free bovine insulin (27 U/mg) came from Hoechst, Frankfurt (Germany). Alloxan and all other reagents were analytical grade products from E. Merck, Darmstadt (Germany).

Animals

Male Sprague-Dawley rats (W. Gassner, Sulzfeld, Germany) weighing 125–150 g and 170–220 g

were used for the in vitro and the in vivo experiments, respectively. Unless stated otherwise the animals had free access to water and a standard laboratory diet (Labortierfutter 57 Z, J. Zahn II, Hockenheim, Germany).

Induction of Diabetes

Alloxan-diabetes was induced by injection of 80–100 mg/kg body weight of alloxan into a tail vein of starved (16–30 h) rats. For one week the animals received 1.5–4 U of bovine depot insulin SC every 12 h, which was sufficient to prevent gross urinary sugar excretion, assessed with Dextrostix (Ames Co., Miles Laboratories, Frankfurt/M., Germany).

Prior to use insulin was withdrawn for at least 36 h and the rats showing plasma concentrations of glucose, ketone bodies and FFA higher than 22 mmol/l, 1.5 mmol/l and 0.8 mmol/l were used. Mean $\pm$ SEM of these measurements were 35.5 $\pm$1.2 mmol/l, 7.1$\pm$0.6 mmol/l and 2.0 $\pm$ 0.1 mmol/l, respectively.

Heart Perfusion

Hearts were prepared under Nembutal anaesthesia (5 mg/kg body weight IP) according to the Langendorff procedure [10], modified by Morgan et al. [11]. After 1–1.5 min of preperfusion in an open system with 8 ml of substrate-free Krebs-Henseleit bicarbonate buffer [12], saturated with O_2 and CO_2 (95:5), hearts were perfused for 30 min in a recirculating system with 17 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, containing 20 g/l bovine serum albumin (Serva, Heidelberg, Germany) (= basal medium) at a constant pressure of 60 mmHg. Before use, the perfusion medium was filtered through a Millipore filter, type SSWP. The flow rate (12–14 ml/min) and frequency of heart contractions (220–260/min) remained constant during the perfusion period.

Determination of Pyruvate Decarboxylation by the Perfused Heart Muscle

Pyruvate decarboxylation was determined using 3,4 $-^{14}C$-labelled glucose (New England Nuclear, Dreieichenhain, Germany), which yields pyruvate and lactate labelled in the carboxyl position. In each experiment 1–2 μCi of labelled glucose (13.5 Ci/mole) were added to the basal medium containing 10 mmol/l glucose.

For radioactivity measurements 0.5 ml of medium was deproteinized with 0.5 ml of 6%

$HClO_4$, neutralized with solid $KHCO_3$, and separated on a Dowex 1 $\times$ 8 column (0.6 $\times$ 8 cm, formate form, 200–400 mesh) at 4°C. On elution at 0.3 ml/min glucose was recovered in the first peak with 15 ml H_2O (3 fractions of 5 ml each), whereas lactate and pyruvate appeared separately on elution with 25 ml of 0.3 mol/l HCOOH (5 fractions), and 25 ml of 6 mol/l HCOOH (5 fractions), respectively. The radioactivity of 2 ml of the combined fractions mixed with 12 ml of Bray's solution [13] was determined in a Mark II liquid scintillation counter and corrected for quenching by internal standard. 98.4–102.3% of the radioactivity placed on the column was recovered in the combined eluates. The specific radioactivity of glucose remained constant during a period of 30 min of perfusion. The specific labelling of lactate and pyruvate was half that of glucose, indicating equilibration of the C_3-pool.

The position of ^{14}C in lactate and pyruvate was examined after permanganate oxidation according to Katz et al. [14]. Ninety-two percent of the label incorporated into lactate and pyruvate could be detected in the carboxyl position after 30 min of heart perfusion with 3,4 $-^{14}C$-glucose.

Measurement of $^{14}CO_2$

$^{14}CO_2$ was trapped in a vessel fitted to the gas outlet of the perfusion chamber containing 10 ml of 25% (v/v) ethanolamine in methanol. $^{14}CO_2$ present as bicarbonate in the medium was collected at the end of the perfusion according to Gliemann [15].

The sum of the radioactivities recovered in glucose, lactate, pyruvate, CO_2 (medium plus gas phase), and in the heart tissue after perfusion amounted to 94–98% of the radioactivity added.

Preparation of Tissue Extracts and Pyruvate Dehydrogenase Determinations

The hearts were removed from anaesthetised rats or from the perfusion chamber with a Wollenberger clamp precooled in liquid N_2, and the frozen tissue was pulverized in a mortar. Homogenization was performed by treating 0.2 g of pulverized heart tissue in 2.6 ml K^+ phosphate buffer (20 mmol/l in 4.3 mol/l glycerol, pH 7.0) at $-10°C$ twice (60 sec each with an intermission of 60 sec) with a high speed tissue disintegrator (Ultra Turrax TP 10 N, Jahnke und Kunkel, Stauffen i. Breisgau, Germany). The temperature did not rise above $-4°C$ during homogenization. PDH activities were measured before and after activation with Mg^{2+} by the dismutation assay described earlier [5].

One unit of PDH activity corresponds to the formation of 1 μmol acetylphosphate per min at 37°C.

Table 1. Effect of insulin on glucose metabolism and on pyruvate dehydrogenase of the perfused diabetic rat heart. For experimental details see Methods

Group	n	Additions	Metabolite changes (μmoles $\times$ g wet wt.$^{-1}$ $\times$ 30 min^{-1})			$^{14}CO_2$ Production μmoles $\times$ g wet wt.$^{-1}$ x min^{-1}	PDH_a Units $\times$ g wet wt.$^{-1}$	% of PDH_{a+b}
			Glucose	Lactate	Pyruvate			
A (control)	22	glucose, 10 mmol/l	−95.7±4.8	+26.2±2.8	+5.7±0.4	1.33±0.05	1.83±0.10	58
B (diabetic)	11	glucose, 10 mmol/l	−47.7±6.2	+18.0±2.3	+4.8±1.6	0.45±0.04	0.38±0.02	13
C (diabetic)	20	glucose, 10 mmol/l insulin, 2 mU/ml	−93.9±6.1	+50.5±3.8	+5.0±0.5	0.77±0.04	1.69±0.11	54
D (diabetic)	10	insulin, 2 mU/ml	+ 1.8±0.2	+18.9±1.2	+1.4±0.3	−	0.50±0.06	15
E (diabetic)	19	deoxyglucose, 10 mmol/l insulin, 2 mU/ml	+ 1.5±0.3	+10.3±1.0	+0.8±0.1	−	2.83±0.20	74

Analytical Methods

Glucose, lactate, pyruvate, acetoacetate and 3-hydroxybutyrate were determined in deproteinized and neutralized perfusate or plasma samples by standard enzymatic procedures [16]. Protein was measured by a modified Biuret procedure [17]. Free fatty acid determinations in plasma samples were carried out by a modified [18] Duncombe procedure [19].

Statistical Evaluation

Results are presented as the mean ($\bar{x}$) ± SEM. Since a normal distribution of the various parameters was not predictable statistical significance was assessed with the Wilcoxon-Mann-Whitney rank test [20].

Results

Effect of Insulin on the Phosphorylation State of PDH in vivo

In the heart muscle from diabetic rats two hours after the injection of 250 mg/kg nicotinic acid SC, 11.8% of total PDH activity (n = 16) was present in the active dephospho form (= PDH_a), when the concentration of plasma FFA was 0.29 ± 0.03 mequ/l (n = 12). Rats kept on insulin until death responded to the same antilipolytic treatment with an increase of PDH_a to 41.6% (n = 13), plasma FFA being 0.22 ± 0.01 mequ/l (n = 11). Total PDH activities were determined to be 6.8 ± 0.4 U/g wet wt. (n = 16) and 8.9 ± 0.4 U/g wet wt. (n = 13) in the diabetic and insulin treated groups, respectively. In the kidney of the diabetic animals PDH_a activity was four times higher than in heart muscle and was not elevated by insulin. Fur-

ther, there was no change of total PDH activity in kidney (5 ± 0.2 U/g wet wt., (n = 16) and 4.5 ± 0.2 U/g wet wt. (n = 12) in the diabetic and the insulin treated group, respectively).

Effect of Insulin on the Phosphorylation State of PDH in vitro

The hearts from diabetic rats perfused for 30 min with glucose had a much lower level of PDH_a than the hearts from normal animals (Table 1), but the same total PDH activity (3.10 ± 0.15 U/g wet wt., n = 8). As further shown in Table 1 insulin added to the incubation medium restored the PDH_a value of the normal control in the diabetic hearts and this effect was dependent on the presence of glucose or deoxyglucose.

The decrease with insulin deficiency in the active PDH form was accompanied by a marked fall of the $^{14}CO_2$ output (Table 1) and by a diminution of glucose uptake and lactate production, by 50% and 31%, respectively. In agreement with an earlier report [33] insulin added to the perfusion medium normalized glucose uptake, markedly increased lactate formation but did not yield the normal rate of $^{14}CO_2$ production (Table 1). Regarding the relationship between pyruvate decarboxylation and PDH_a activity extractable from heart muscle, the present study took advantage of the fact that ^{14}C-glucose labelled in the C_3 and C_4 positions yields almost exclusively carboxyl-^{14}-C-pyruvate and lactate. As no isotope dilution of the C_3-pool was measurable during the 30 min perfusion period and $^{14}CO_2$ redistribution, in view of the low pyruvate carboxylase activity in heart muscle [37], appears to be negligible, it seemed reasonable to estimate the flux of pyruvate through the PDH step from the $^{14}CO_2$ formed on the basis of one mole of 3,4-^{14}C-glucose yielding two moles of 1-^{14}C-pyruvate.

According to Table 1 $^{14}CO_2$ production by hearts from normal and diabetic rats corresponded to 75% and 100% respectively, of PDH_a activity. In the presence of insulin (group C), however, the rate of $^{14}CO_2$ formation apparently accounted only for 45% of the PDH_a activity.

Discussion

In an earlier study an inverse relationship between the levels of plasma FFA and PDH_a in heart muscle and kidney from intact rats was demonstrated [5]. In starved rats, for example, PDH_a of heart muscle rose from 7% to 65% and that of kidney from 15% to 78% when the concentration of plasma FFA was normalized by nicotinic acid treatment [5]. In the present study on diabetic rats the amount of heart muscle PDH_a was much lower than expected [5] from the plasma FFA concentrations after nicotinic acid treatment, while that of the kidney approached the normal value. As diabetic animals injected with insulin showed a marked increase of PDH_a in heart muscle but not in kidney it is reasonable to conclude that insulin is involved in the regulation of PDH interconversion solely in the former organ. This view is further substantiated by our studies conducted on the perfused heart. These experiments clearly showed that insulin added *in vitro* was capable of restoring the level of PDH_a of diabetic hearts to normal values (Table 1). This result and the values for PDH_a in diabetic and normal heart muscle differ from those reported by Kerbey et al. [21], who found only 25–30% and 1.5% of total enzyme activity present in the active form in perfused hearts from normal and diabetic rats. Moreover, insulin failed to restore normal PDH_a levels in their preparations from diabetic rats. The reason(s) for these discrepancies are not clear; however, the experimental conditions with respect to age and strain of the animals, perfusion time and medium glucose concentration were different. Further, our animals had been kept on insulin for one week after alloxan injection and only those displaying severe ketoacidosis and hyperglycaemia after insulin withdrawal were used.

As to the mechanism of insulin action on PDH interconversion the hormone seems to act *via* PDH kinase rather than *via* PDH-phosphatase, since the activity of the latter enzyme proved to be normal in diabetes [36].

From the fact that insulin in heart muscle, like in adipose tissue [24], affects PDH_a only in the presence of glucose or deoxyglucose, it appears that its effect, at least in part, could be explained by a lowering of the mitochondrial ATP/ADP ratio which may itself result from the increased consumption of

cytosolic ATP for sugar phosphorylation and/or anabolic processes, in particular glycogen synthesis [33]. However, a number of additional factors, such as pyruvate concentration, the ratios of NADH/NAD and of acetyl-CoA/CoA, have been demonstrated in studies with isolated rat heart mitochondria to act on PDH interconversion [21, 27–30]. Pertaining to pyruvate, insulin neither causes an accumulation in the perfusion medium (Table 1) nor in the heart tissue [31, 32]. Also with respect to ATP and ADP insulin did not change the total cellular contents of perfused hearts from normal and diabetic animals [34, 35]. These findings, however, do not exclude the possibility that opposite metabolite changes may occur in the mitochondrial and cytosolic spaces. That this can happen under special conditions has been demonstrated recently with isolated rat liver cells [38]. Unfortunately, no method is available at present to study the subcellular distribution of metabolites in heart cells.

As to the question of feedback control being operative on PDH in heart muscle the data presented in groups A and B of Table 1 are compatible with the view that the enzyme is not subject to great restraint in the intact cell. The discrepancy apparent in group C could, at least in part, be explained by the fact that the PDH activity prevailing at the end of the perfusion time has been compared with a rate of $^{14}CO_2$ formation averaged over the 30 min period. As insulin markedly increased PDH_a activity during perfusion, the average enzyme activity must have been lower than that given in Table 1, group C. Thus it appears that in the isolated perfused heart muscle feedback control of PDH is less pronounced than in isolated heart mitochondria from normal fed rats since, during state 3 respiration, the rate of pyruvate decarboxylation accounts only for about 50% of PDH_a activity [30].

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