Effect of starvation and insulin in vivo on the activity of the pyruvate dehydrogenase complex in rat skeletal muscles

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The in vivo responses of pyruvate dehydrogenase (PDH) complex to starvation and insulin was assessed in heart, diaphragm and red quadriceps muscle. PDH complex activity was decreased by starvation (3.4–10.2-fold), the magnitude of change depending on muscle type. Insulin increased PDH activity in all muscle types. In fed rats, this effect was relatively small (1.25–1.29-fold). In starved rats there were effects in heart (4.3-fold) and red quadriceps (1.7-fold) but no effect in diaphragm. These results demonstrate that PDH complex in different groups of muscle has different insulin sensitivity (particularly in tissues from starved animals).

Pyruvate dehydrogenase complex; Skeletal muscle; Starvation; Insulin

1. INTRODUCTION

The regulation of muscle glucose metabolism is thought to be of critical importance to the maintenance of euglycemia [1]. Thus an inability of muscle to dispose adequately of glucose after challenge with a meal may impair glucose tolerance. Conversely, during periods of starvation it is important for muscle carbohydrate oxidation to be inhibited for the conservation of glucose. For these adaptations to occur the enzymes responsible for the storage and oxidation of glucose in skeletal muscle must be sensitive to the glucoregulatory hormones.

Glucose oxidation to carbon dioxide in muscle is dependent on the activity of the pyruvate dehydrogenase (PDH) complex. The regulatory properties of skeletal muscle PDH complex are similar to those of this complex in other tissues [2,3]. Thus, phosphorylation of the enzyme, catalysed by PDH kinase inactivates the complex, while dephosphorylation, catalysed by PDH phosphatase, reac-

tivates it. The total amount of the PDH complex in the tissue (sum of active and inactive forms) does not change in the short term; only the percentage of the complex in the active form (%PDHa) fluctuates [3–5]. Skeletal muscle PDH complex contains three serine residues that are available for phosphorylation but occupancy of only one site is necessary for the inactivation of the enzyme [3]. From studies using rat heart, it has been postulated that phosphorylation of the other two sites may provide a mechanism for inhibiting PDH phosphatase and thus delay reactivation of the complex [6].

The effects of starvation or insulin on PDH complex activity have not been studied as extensively in skeletal muscle as they have in heart or adipose tissue. This is due to difficulties with extraction and assay of the enzyme from this highly fibrous tissue. Indeed, reports of changes in %PDHa have been variable both with regard to estimates of total PDH complex activity and %PDHa (compare [4,7–9]). Despite this it has been reported that %PDHa is reduced by starvation in gastrocnemius muscle [4,10] and that muscle PDH complex activity is increased by insulin

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[11]. In addition, an attempt has been made to determine the relationship between insulin dose and the magnitude of the PDH response [12]. However, some workers [5] have been unable to detect an effect of insulin on skeletal muscle %PDHa.

Such inconsistencies in the literature make assessing the response of the enzyme to hormonal and nutritional stimuli difficult. It is also becoming increasingly clear that the different types of skeletal muscle exhibit a wide range of responses of glucose metabolism to insulin [13]; thus it is essential for studies on skeletal muscle PDH complex regulation to examine more than one muscle group. A further factor that should be considered in assessing the response of PDH complex to insulin is the nutritional status of the animal, since the multi-site phosphorylation occasioned by starvation [6] may reduce the effects of glucoregulatory hormones.

In the present study, we have determined PDH complex activity in mitochondria isolated from skeletal muscles. We have measured the effect of starvation and/or insulin administration on the activity of PDH complex in two types of skeletal muscle; diaphragm, a continually working aerobic muscle and red quadriceps, an aerobic muscle which would be mainly at rest during the course of these experiments. Since the effects of starvation [14] and insulin [15] on PDH activity in heart and brown adipose tissue, respectively, are well documented, these tissues were also studied for comparison.

2. MATERIALS AND METHODS

Male albino Wistar rats (200–250 g) were kept in the departmental animal house and allowed free access to food (Mouse and Rat Kubes, Allied Feeds, Rhodes, NSW, Australia) and water. Animals were studied at 10.00 a.m., 3 h (fed) or 48 h (starved) after the removal of food. The effects of insulin were tested by injecting the animals intraperitoneally with a mixture of insulin (1 U/kg) and glucose (1 g/kg) in approx. 500 μl of 0.9% saline; the glucose being given to prevent hypoglycemia. Control animals received a similar volume of saline. 30 min later the animals were anesthetised (Nembutal, 60 mg/kg body wt, intraperitoneal injection), a blood sample (100–200 μl) taken from the heart for estimation of blood glucose concentration [8], and tissues were excised. Insulin was assayed by double antibody radioimmunoassay using rat insulin standards.

Tissues (200–500 mg depending on type) were disrupted (Polytron homogeniser, setting no.2–3) in sucrose buffer (0.25 M sucrose/5 mM Tris-HCl/2 mM EGTA, pH 7.5) con-taining 50 mM sodium fluoride and 10 mM sodium dichloroacetate (as inhibitors of PDH kinase and PDH phosphatase). Mitochondria were prepared by differential centrifugation [16] and divided into two portions. One portion, used for the determination of the PDH complex activity at time of sacrifice (PDHa), was pelleted by centrifugation and immediately frozen in liquid nitrogen. The other portion of mitochondria was washed free of sodium fluoride by dilution (100 vols of sucrose medium), followed by centrifugation. These mitochondria were then incubated (30 min at 30°C) in KCl medium (120 mM KCl/20 mM Tris-HCl/5 mM potassium phosphate/2 mM EDTA, pH 7.4) containing 10 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP, an uncoupler of oxidative phosphorylation). This incubation depletes mitochondrial ATP concentration and allows complete conversion of inactive PDH complex to active PDH complex by PDH phosphatase. These mitochondria were pelleted by centrifugation (10000 × g), frozen in liquid nitrogen and used to obtain an estimate of PDH total. Mitochondria were extracted for assay of enzymes and protein concentration by alternate thawing (30°C) and freezing (liquid nitrogen, three times) in extraction buffer (50 mM potassium phosphate/10 mM EGTA/2 mM dithiothreitol, pH 7.0) containing 1% Triton X-100.

PDH complex activity was assayed spectrophotometrically in extracts of mitochondria by following the production of acetyl CoA from pyruvate and CoA [14]. Citrate synthase activity and protein concentration were determined spectrophotometrically [14] as indications of mitochondrial recovery and purity. Citrate synthase activity was also determined in extracts of frozen tissue. In these experiments, animals were anesthetised, tissues excised and frozen in tongs cooled in liquid nitrogen. Samples of these tissues (70–150 mg) were extracted (Polytron, setting no.5) in 3–4 ml extraction buffer (as above minus Triton X-100). Diluted samples of whole homogenate were used for citrate synthase determination.

3. RESULTS AND DISCUSSION

The plasma glucose concentrations in samples withdrawn from the heart 30 min post-injection were 6.0 ± 0.6 mM (saline injection) and 12.6 ± 1.3 mM (insulin/glucose injection); therefore the injection of the insulin/glucose mixture did not cause hypoglycemia. This is important as hypoglycemia causes the release of insulin counterregulatory hormones (e.g. adrenaline, glucagon) which may effect PDH complex activity [17]. The glucose levels achieved with the glucose/insulin injection are similar to those obtained post-prandially. In addition, while hyperglycemia alone does increase tissue glucose uptake, it may not alter PDH complex activity [15]. Insulin levels were 119 ± 9, 34 ± 5 and 2140 ± 325 μU/ml in fed, starved and insulin injected animals, respectively.

For each tissue, total PDH complex activity was unchanged regardless of the treatment of the

animals (i.e. insulin injection or starvation). Values of total mitochondrial PDH complex activity (table 1) obtained for heart and BAT were comparable with previously published data [14,18]; thus the values obtained for diaphragm (70.2 ± 2.8 mU/mg protein) and red quadriceps (88.9 ± 3.6 mU/mg protein) can be viewed with confidence. In addition, these values are similar to those obtained by Fuller and Randle [3] (76.4 mU/mg protein) and Ashour and Hansford [2] (approx. 97 mU/mg protein) for mitochondria derived from the mixed hindlimb muscles (using trypsin incubation to maximize yield). Similarly mitochondrial citrate synthase activity was constant for a given tissue (table 1) and was independent of either treatment of the animal, or whether the mitochondria were used for determination of total or active PDH complex activity (not shown).

Much of the current knowledge concerning the in vivo regulation of skeletal muscle PDH complex activity has been obtained by assaying extracts of frozen tissue. This method may not be wholly quantitative because extraction of the enzyme may be variable and incomplete. Accurate spectrophotometric assay of PDH complex requires the preparation of concentrated muscle tissue extracts and centrifugation (to reduce turbidity) which may result in the loss of PDH complex to the pellet. Conversion of inactive PDH complex to active PDH complex (for estimation of total PDH complex activity) requires prior preparation of PDH phosphatase and lengthy incubation which may expose PDH complex to lysosomal proteases. It should also be noted that although it would be possible to prepare muscle tissue extracts in the presence of interconversion inhibitors (to obtain an estimate of PDHa) it would be impractical to wash these inhibitors out of the extracts for subsequent reactivation of PDH complex. Using the results for whole tissue citrate synthase activity (table 1) coupled with the computed PDH total/CS ratio, it is possible to calculate that the total PDH complex activities of each of the tissues on a U/g wet wt basis are 16.2 (heart), 4.8 (diaphragm), 5.2 (red quadriceps) and 16.0 (BAT). Though it should be pointed out that the use of only one mitochondrial marker enzyme to calculate these values may have some inherent errors, the values obtained are greater than those found using the whole tissue extraction method and may indicate incomplete extraction or reactivation of the enzyme by that method.

In muscle tissues from fed animals, %PDHa was about 50% (table 2). These values are similar to those obtained by Stansbie et al. [7] (49%) but differ from those obtained by ourselves and others using gastrocnemius or mixed hindlimb muscles – e.g. Hennig et al. [4] (15%), Hagg et al. [10] (22%), and Caterson et al. [8] (68%).

PDH complex activity was significantly decreased by starvation in all the muscle groups but the magnitude of change depended on the muscle type (table 2). Thus the effects of starvation were most marked in the heart: 10.2-fold decrease as compared to diaphragm (3.4-fold) and red quadriceps (4.7-fold). Brown adipose tissue PDHa was also decreased by starvation (4.4-fold). Decreased PDHa may be a consequence of increased PDH kinase activity due to elevations in the concentrations of the products of fatty acid oxidation [14]. In addition, starvation may increase the activity of PDH kinase in a stable manner, as has been shown in preparations of hindlimb muscle mitochondria [3].

Insulin significantly increased PDH activity in all muscle tissues from fed rats (table 2), but the magnitude of these effects was relatively small (25–29%) in contrast to the marked stimulatory effect of insulin on BAT PDH activity (134%). This may mean that the PDH complex in muscle and BAT exhibits different insulin sensitivities, or that the time period of exposure to insulin has to be greater in the skeletal muscles than BAT for the same effect to be observed. The time of exposure and dose of insulin used in these experiments have previously been shown to be sufficient to elevate PDH complex activity in white adipose tissue [19].

In starved animals, there was a substantial stimulation of PDH complex by insulin in heart and BAT (4–5-fold) and a smaller effect was also apparent in the red quadriceps (1.7-fold) (table 2). However, there was no effect of insulin on PDH complex activity in the diaphragm (table 2). This demonstrates that different groups of muscle have different insulin sensitivity and future studies must take these differences into account. Heterogeneity between the different muscle groups with respect to the effect of insulin on PDH complex activity may be related, in part, to different rates of fatty acid oxidation and/or whether the muscle is work-

Table 1

Activities of total pyruvate dehydrogenase (PDH) complex and citrate synthase in rat tissue extracts and mitochondria

	Citrate synthase activity		Total PDH complex activity	
	U/mg mitochondrial protein	U/g wet wt tissue	mU/mg mitochondrial protein	PDHt/citrate synthase (mitochondrial mU/U)
Heart	1.06 ± 0.03	182 ± 3	94.1 ± 3.5	89.2 ± 2.9
Diaphragm	0.88 ± 0.02	59 ± 3	70.2 ± 2.8	80.7 ± 2.8
Red quadriceps	1.03 ± 0.02	59 ± 3	88.9 ± 3.6	88.3 ± 3.5
BAT	1.69 ± 0.06	211 ± 13	129.7 ± 4.3	75.6 ± 1.5

Mitochondria were prepared from rat tissues in the presence of inhibitors of PDH complex interconversion. To effect full dephosphorylation of the PDH complex within the mitochondria, a portion of the mitochondria was washed free of the interconversion inhibitors and then incubated in the presence of 10 μM CCCP (as described in section 2). PDH complex activity was assayed in extracts of these mitochondria to obtain an estimate of total PDH complex activity (PDHt). Citrate synthase activity was determined in both extracts of mitochondria and extracts of frozen tissue. The results are the means ± SE of 22 observations for mitochondrial determinations and ± SE of 6 observations for determinations in whole tissue extracts

ing or at rest. These experiments do not differentiate between the direct effects of insulin on muscle tissue and the antilipolytic effects of insulin which would decrease the availability of free fatty acids.

In starved animals, insulin did not increase skeletal muscle PDH complex activity to fed values. Whether PDH complex activity would have increased further if the insulin were allowed more than 30 min to act was not determined, but it has been shown that skeletal muscle PDH complex activity in starved animals returns to fed values after 2.5 h on a hyperinsulinemic-euglycemic clamp

[20]. However, it remains a possibility that starvation renders the PDH complex resistant to full reactivation following a short-term stimulus with insulin, especially in the diaphragm. No such delay of glycogen synthase reactivation after starvation has been described, and it is tempting to speculate that this provides a mechanism by which glucose is channeled into storage rather than oxidation upon refeeding after starvation.

The present study has shown that the effect of insulin in stimulating PDH complex activity in skeletal muscle is not the same in all muscle types,

Table 2

Effects of starvation and insulin in vivo on the activity of pyruvate dehydrogenase (PDH) complex in rat

Animal	PDH complex activity							
	Heart		Diaphragm		Red quadriceps		BAT	
	mU/mg	% of total	mU/mg	% of total	mU/mg	% of total	mU/mg	% of total
Fed control	52.8 ± 2.6	54.1 ± 2.6	33.0 ± 2.2	45.3 ± 2.1	51.1 ± 4.8	55.9 ± 2.9	47.1 ± 3.8	34.9 ± 3.9
Fed + insulin	63.8 ± 2.1**	67.7 ± 2.2**	42.7 ± 2.2**	58.6 ± 2.8**	70.3 ± 4.6**	71.3 ± 3.5**	101.3 ± 2.6**	81.7 ± 1.0**
Starved control	5.5 ± 1.4*	5.3 ± 1.1*	9.3 ± 1.4*	13.5 ± 2.2*	9.8 ± 1.2*	12.2 ± 1.6*	10.7 ± 1.2*	8.0 ± 0.6*
Starved + insulin	19.3 ± 1.9**	22.9 ± 2.8**	8.7 ± 0.2	12.6 ± 1.3	17.3 ± 1.9**	20.6 ± 2.4**	48.4 ± 1.9**	37.5 ± 1.4**

Rats (fed or 48 h starved) were injected with approx. 500 μl of a mixture of insulin (1 U/kg) and glucose (1 g/kg) in 0.9% saline. Control animals received a similar volume of saline. Thirty minutes later the animals were anesthetised and tissues excised. Mitochondria were prepared from the tissues in the presence of inhibitors of PDH complex interconversion. After preparation, half of the mitochondria were frozen and extracted for assay of PDH complex activity (PDHa). The other half were washed free of the interconversion inhibitors and used to obtain an estimate of total PDH complex activity (table 1). The results are the means ± SE of 4–8 observations. * $P < 0.001$ for effect of starvation; ** $P < 0.05$ for effect of insulin/glucose

and that starvation may reduce or delay this effect in some muscle groups. One problem with this study was that the simple injection method did not allow determination of the actual concentrations of insulin and glucose in the plasma during the course of the experiments. The relationship between muscle PDH complex activity and insulin concentration would be better studied in animals maintained under a euglycemic clamp. This has been attempted in humans by Mandarino et al. [12] using the tissue extract method for estimation of muscle PDH complex activity. It may be useful to confirm and extend the insulin dose-response experiments using the alternative method for PDH complex estimation described in the present study, taking into account the nutritional status of the animals and examining more than one muscle group.

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