Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart

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This study investigated whether conditions known to alter the activity and phosphorylation state of the pyruvate dehydrogenase complex have specific effects on the levels of isoenzymes of pyruvate dehydrogenase kinase (PDK) in rat heart. Immunoblot analysis revealed a remarkable increase in the amount of PDK4 in the hearts of rats that had been starved or rendered diabetic with streptozotocin. Re-feeding of starved rats and insulin treatment of diabetic rats very effectively reversed the increase in PDK4 protein and restored PDK enzyme activity to levels of chow-fed control rats. Starvation and diabetes also markedly

increased the abundance of PDK4 mRNA, and re-feeding and insulin treatment reduced levels of the message to that of controls. In contrast with the findings for PDK4, little or no changes in the amounts of PDK1 and PDK2 protein and the abundance of their messages occurred in response to starvation and diabetes. The observed shift in the relative abundance of PDK isoenzymes probably explains previous studies of the effects of starvation and diabetes on heart PDK activity. The results indicate that control of the amount of PDK4 is important in long-term regulation of the activity of the pyruvate dehydrogenase complex in rat heart.

INTRODUCTION

Regulation of the activity of the pyruvate dehydrogenase complex by covalent modification is of great importance to the control of glucose and lactate oxidation by well-oxygenated tissues. Inactivation of the complex by phosphorylation by its intrinsic kinase (pyruvate dehydrogenase kinase; PDK) limits the use of glucose by peripheral tissues and conserves three carbon compounds for glucose synthesis by the liver and kidney. Activation of the complex by dephosphorylation by its specific phosphatase (pyruvate dehydrogenase phosphatase) increases the availability of acetyl-CoA for lipogenesis in the liver and adipose tissue and for complete oxidation by the citric acid cycle in the heart and many other tissues.

Starvation and experimentally induced diabetes cause a marked decrease in the activity state of the pyruvate dehydrogenase complex (percentage in the active dephosphorylated state) in most but not all tissues of the rat (reviewed in [1–3]). This is physiologically advantageous in the starved state since conservation of three carbon compounds for gluconeogenesis provides glucose for tissues dependent on this substrate, e.g. the brain, and it also indirectly conserves body protein by decreasing the demand for gluconeogenic amino acids. On the other hand, inhibition of the complex exacerbates the diabetic state by inappropriately sparing glucose and gluconeogenic substrates from complete oxidation in the face of abundant levels of glucose and other oxidizable fuels in the blood.

Both short- and long-term regulatory mechanisms are involved in setting the activity state of the pyruvate dehydrogenase complex. The former include activation of PDK activity by products (acetyl-CoA and NADH) of fatty acid oxidation and the pyruvate dehydrogenase complex, as well as inhibition of PDK by pyruvate, derived from the glycolytic pathway or circulating lactate and alanine (reviewed in [4–6]). Long-term mechanisms include a stable increase in PDK activity that is

independent of short-term effects of small molecule effectors [7–16]. Although considerable study of the purified complex has resulted in some understanding of short-term regulation, the molecular mechanisms responsible for long-term control have remained elusive.

The recent finding that multiple PDK isoenzymes exist in cells of higher eukaryotes [17–21] has raised intriguing new possibilities for regulation of the pyruvate dehydrogenase complex. The isoenzymes exhibit unique tissue expression patterns [17–21] and are differentially responsive to regulation by pyruvate, acetyl-CoA and NADH [19,21]. The physiological significance of these findings is that the activity state of the pyruvate dehydrogenase complex is most likely a function of the relative amounts of the PDK isoenzymes present in a particular tissue in a given metabolic condition. The present study was conducted to test the hypothesis that starvation and diabetes may increase the amount of one or more of the PDK isoenzymes in rat heart.

MATERIALS AND METHODS

Materials

Protamine-zinc insulin was obtained from Anthony Products. ¹²⁵I-Protein A was obtained from ICN Biochemicals. Radioactive nucleotides were from England Nuclear Research Products. All other chemicals were from Sigma Chemical Company. The random-primed DNA labelling kit for labelling cDNA probes was from Boehringer-Mannheim GmbH. Arylamine acetyltransferase was prepared from pigeon liver by a procedure basically described previously [22] except that hydroxyapatite and sizing column chromatography were not used. Recombinant PDK1, PDK2 and PDK4 were expressed in *Escherichia coli* and purified by chromatography on either His-Bind (Novagen) or Talon NX resin (Clontech) as described in the preceding paper [21].

Abbreviations used: PDK, pyruvate dehydrogenase kinase; E1 α , α -subunit of the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex.

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Animals

Male Wistar rats (Harlan Industries) with initial body weights of about 200 g were housed in a temperature- and light-controlled room. They were fed Purina Rodent laboratory chow diet *ad libitum* for several days before initiation of each experiment. For studies of the effects of starvation, food was removed from the cages 48 h before the animals were killed. For studies of the effects of diabetes, rats starved overnight were injected intravenously with either streptozotocin (65 mg/kg body weight) or physiological saline. Free access to food and water was provided until the animals were killed. Half of the diabetic animals were killed 48 h after injection of streptozotocin. The remainder were started 48 h after the streptozotocin injection on injections of 1–2 units of protamine-zinc insulin/100 g body weight every 12 h and killed 48 h later.

Enzyme assays

Mitochondria were isolated from rat hearts as described previously by Kerbey et al. [23]. Protein was determined by the Bradford method [24] using Bio-Rad protein assay dye reagent. To activate the pyruvate dehydrogenase complex completely via dephosphorylation with endogenous pyruvate dehydrogenase phosphatase, freshly isolated mitochondria were incubated for 25 min at 30 °C in medium (pH 7.4) containing 120 mM KCl, 20 mM Tris/HCl, 5 mM potassium phosphate, 2 mM EGTA and 10 μ M carbonyl cyanide *m*-chlorophenylhydrazone [7,12]. The mitochondria were recovered from this incubation medium by centrifugation (10000 g; 10 min) and extracted by freeze/thawing three times in extraction buffer (50 mM potassium phosphate, pH 7.5, 10 mM EGTA, 2 mM dithiothreitol, 2% bovine serum and 0.5% Triton X-100) [8]. Activity of the pyruvate dehydrogenase complex in this extract was assayed spectrophotometrically at 30 °C by coupling the generation of acetyl-CoA to the acetylation of *p*-(*p*-aminophenylazo)benzenesulphonic acid by arylamine acetyltransferase [25] in assay buffer [0.1 M Tris/HCl, pH 7.8, 0.5 mM EDTA, 1 mM MgCl₂, 0.375 unit/ml lipoamide dehydrogenase, 5 mM 2-mercaptoethanol, 1 mM thiamine diphosphate, 0.5 mM NAD⁺, 0.1 mM CoA, 0.04 mM *p*-(*p*-aminophenylazo)benzenesulphonic acid, 1 mM pyruvate and excess arylamine acetyltransferase (determined empirically)]. PDK activity was assayed at 30 °C in another assay buffer (50 mM potassium phosphate, pH 7.5, 1 mM MgCl₂, 5 units/ml creatine kinase, 10 mM phosphocreatine and 5 mM KF). The reaction was started by adding mitochondrial extract (0.1–0.2 mg of protein) and 1 mM ATP to the assay buffer. Samples taken at four time points were diluted with the assay buffer for the pyruvate dehydrogenase complex and the remaining activity of the complex was determined by the arylamine acetyltransferase-coupled assay. The activity of PDK is expressed as the first-order rate constant of inactivation of the pyruvate dehydrogenase complex over the time of incubation with ATP.

Immunoblotting analysis

Mitochondria were solubilized by boiling in 50 mM Tris/HCl, pH 6.8, containing 2% SDS, 0.1 M dithiothreitol, 0.1% Bromophenol Blue, 10% glycerol, 1 mM benzamidine, 100 μ g/ml trypsin inhibitor, 1 μ g/ml aprotinin, 0.1 mM TosLysCH₂Cl, 1 μ M leupeptin and 1 μ M pepstatin A. SDS/PAGE of the extracts and Western blots were conducted by the protocol described previously [26]. Polyclonal antisera against recombinant PDK1, PDK2 and PDK4 were generated in rabbits by a standard protocol [27]. The antibodies for each of these

isoenzymes were specific and did not cross-react with the other PDK isoenzymes under the loading and detection conditions used for Western-blot analysis. We were not successful in producing a specific antibody against PDK3 that would detect the small amounts of this protein expressed in heart cells. Antibody–antigen complexes were visualized with ¹²⁵I-labelled Protein A and autoradiography. Relative densities of bands on the films were determined with a Bio-Rad GS-670 Imaging Densitometer equipped with Molecular Analyst 2.1 software. The amounts of mitochondrial extracts loaded on to the gel were varied to establish that relative densities of the bands corresponding to the PDK isoenzymes were linear with concentration. For quantification of the amount of a PDK isoenzyme, four concentrations of the corresponding recombinant PDK isoenzyme were included on the blot to establish a standard curve. Although concentration ranges of linearity and slopes varied, conditions were established under which linear standard curves were obtained for each of the recombinant PDK isoenzymes.

Northern-blot analysis

Total RNA was extracted from freeze-clamped hearts with Tri Reagent™ according to the instructions of the manufacturer (Sigma Bioscience). Northern blotting was conducted by the protocol described previously [18]. ³²P-labelled cDNAs of rat PDK1 [17], PDK2 [18] and PDK4 [21] were used as probes for the hybridizations. A labelled oligonucleotide designed according to the sequence of 28 S rRNA [28] was used to normalize RNA loading to gels. Quantification was accomplished by densitometry as described above for immunoblot analysis.

Statistical analysis

Data were analysed for statistical significance by Student's *t* test for unpaired samples.

RESULTS

Effects of starvation on mitochondrial content of PDK isoenzymes in rat heart

In keeping with previous findings [7–16], we consistently find a 2–3-fold increase in PDK activity in hearts of rats starved for 48 h. To assess whether this effect of starvation is due to an increase in amounts of the PDK isoenzymes, extracts of heart mitochondrial preparations from fed and starved rats were subjected to Western-blot analysis (Figure 1). A greater than 3-fold increase in the amount of PDK4 protein in heart mitochondria of starved rats is the most remarkable finding. In contrast, starvation was without significant effects on the relative amounts of the PDK1 isoenzyme, the PDK2 isoenzyme and the E1 α subunit of the pyruvate dehydrogenase complex (Figure 1). Re-feeding starved rats for 48 h was found in a separate experiment to reverse the increase in PDK activity caused by starvation and to decrease markedly the amount of PDK4 protein (results not shown).

Effects of streptozotocin-induced diabetes and insulin treatment of diabetic rats on mitochondrial content of PDK isoenzymes in rat heart

An acute diabetic state was induced by streptozotocin in the rats used in this study as evidenced by increased blood glucose levels (control rats, 116 $\pm$ 6 mg/dl; streptozotocin-injected rats, 436 $\pm$ 22 mg/dl; means $\pm$ S.E.M; four rats/group) and the presence of readily detectable amounts of glucose and ketone bodies in the

Figure 1 Effect of starvation on the mitochondrial content of PDK1, PDK2 and PDK4 in rat heart

Proteins extracted from mitochondria (5 μ g of protein per lane) were separated by SDS/PAGE and transferred to a nitrocellulose membrane for Western-blot analysis. Membranes were probed with polyclonal antibodies for PDK1, PDK2, PDK4 and the E1 α of the pyruvate dehydrogenase (PDH) complex as indicated. Data for three control (chow-fed) rats and three rats starved for 48 h are shown.

Figure 2 Effect of streptozotocin-induced diabetes and insulin treatment of rats with diabetes on the content of PDK1, PDK2 and PDK4 in heart mitochondria

The procedure for Western-blot analysis is described in the legend for Figure 1. Data are given for four control (sham-treated, chow-fed) rats, four diabetic rats and four insulin-treated diabetic rats.

urine (results not shown). The stable increase in PDK enzyme activity previously reported to be induced in rat heart by the diabetic state was also found in the present study (control rats, $0.67 \pm 0.10 \text{ min}^{-1}$; diabetic rats, $1.71 \pm 0.09 \text{ min}^{-1}$; $P < 0.05$; means $\pm$ S.E.M.; four rats/group). As found for starved rats, no effect of the diabetic state on the amount of the E1 α subunit of the pyruvate dehydrogenase complex in heart mitochondria was apparent by Western-blot analysis (Figure 2). No effect of diabetes was likewise found for the amount of PDK2 protein. However, a modest increase (1.35-fold) in PDK1 protein and a large increase (greater than 10-fold) in PDK4 protein was induced by diabetes (Figure 2).

To determine whether the effects of diabetes on PDK activity and PDK4 expression could be reversed by insulin, rats previously made diabetic with streptozotocin were treated with insulin twice a day for 2 days. That all rats were in a severely diabetic state before initiation of insulin therapy was established by blood glucose determination ($444 \pm 19 \text{ mg/dl}$; means $\pm$ S.E.M.; four rats/group) and semiquantitative urine glucose and ketone body analysis (results not shown). Blood glucose levels of the treated animals were significantly reduced ($204 \pm 35 \text{ mg/dl}$; $P < 0.001$ relative to untreated rats; means $\pm$ S.E.M.; four rats/group) and urine glucose and ketone bodies were undetectable (results not

Figure 3 Effect of starvation on PDK2 and PDK4 mRNA levels in rat heart

Total RNA (15 μ g) prepared from hearts from three control and three 48 h starved rats was separated by denaturing gel electrophoresis and transferred to a nylon membrane. PDK2 and PDK4 mRNA was detected with ^{32}P -labelled cDNAs. mRNA sizes were 2.4 kb for PDK2 and 3.9 kb for PDK4. rRNA (loading control) was detected with ^{32}P -labelled antisense 28 S rat rRNA.

shown). This treatment of diabetic rats with insulin lowered PDK activity of their heart mitochondria to values comparable with that of normal chow-fed rats (diabetic rats, $1.71 \pm 0.09 \text{ min}^{-1}$; insulin-treated diabetic rats, $0.82 \pm 0.10 \text{ min}^{-1}$; $P < 0.05$; means $\pm$ S.E.M.; four rats/group). Insulin treatment had no effect on PDK1 protein levels and caused a modest decrease on PDK2 protein (Figure 2). The most remarkable change was again seen in PDK4 protein. Insulin treatment of diabetic rats decreased the level of this protein dramatically, reducing it back to a level comparable with that seen in the control rats (Figure 2).

Effect of starvation and diabetes on the relative amounts of the PDK isoenzymes in rat heart

Standard curves generated by loading various amounts of recombinant PDK proteins on gels for Western-blot analysis (see the Materials and methods section) were used to quantify the relative abundance of the PDK isoenzymes in hearts from control, starved and diabetic animals. Ranges in which densities of the protein bands increased linearly with amount of protein loaded was established for each of the recombinant proteins. Care was taken to ensure that the densities of PDK isoenzyme bands obtained with mitochondrial extracts were within this range of the standard curves established with the corresponding recombinant proteins loaded on to the same gel. From the resulting analysis, the relative abundance of PDK4 was $29 \pm 2\%$ of the total PDK protein in heart mitochondria from three control rats. The corresponding values for PDK4 abundance in heart mitochondria of three starved and three diabetic rats were 55 ± 1 and $75 \pm 2\%$ respectively.

Effects of starvation, diabetes and insulin treatment on mRNA levels

Starvation of rats for 48 h was without significant effect on mRNA levels for PDK2 (Figure 3) and PDK1 (results not shown). In contrast, a dramatic increase (greater than fourfold) in the mRNA for PDK4 was induced by starvation (Figure 3). Diabetes likewise caused no change in the messages for PDK2 (Figure 4) and PDK1 (results not shown), but induced a very large increase in PDK4 mRNA (Figure 4). Treatment of the diabetic rats with insulin reduced the PDK4 mRNA level back to that observed in hearts of control animals (Figure 4).

Figure 4 Effect of streptozotocin-induced diabetes and insulin treatment of rats with diabetes on PDK2 and PDK4 mRNA levels in rat heart

Total RNA (5 µg) prepared from the hearts of three control, three diabetic and three insulin-treated diabetic rats were subjected to Northern-blot analysis as described in the legend to Figure 3. mRNA sizes were 2.4 kb for PDK2 and 3.9 kb for PDK4.

Figure 5 Time course of changes in relative abundance of PDK4 mRNA during starvation followed by re-feeding

Food was removed from cages of all rats at 07:00 h. Re-feeding chow diet was initiated after 48 h of starvation as indicated. Three rats were killed at each point and the total RNA prepared from these animals was pooled for Northern-blot analysis. Abundance of PDK4 mRNA at each time point was quantified and normalized to that of rats killed at the zero time point.

The time course of the effects of starvation followed by re-feeding on the abundance of PDK4 mRNA is shown in Figure 5. The most dramatic increase occurred between 12 and 24 h of starvation, with perhaps some additional increase after the rats had been without food for 48 h. A substantial decrease in PDK4 message level occurred 6 h after the rats had been re-fed an ordinary laboratory chow diet.

DISCUSSION

The fact that several molecular mechanisms (reviewed in [4–6]) have evolved to control the activity of the pyruvate dehydrogenase complex provides strong evidence for the importance of regulation of the activity of this complex to the fuel economy of the body. Maintaining blood glucose levels during fasting depends on restriction of the activity of this enzyme in the major oxidative tissues of the body. Although much remains to be learned about each of the regulatory mechanisms for the complex, the long-term control mechanism in which a stable increase in

PDK activity occurs in various tissues in response to starvation and diabetes [7–16] has been among the most recalcitrant to explain at the molecular level. However, the discovery that four PDK isoenzymes exist in mitochondria [17–21] raised the exciting possibility that increased amounts of one or more of the PDK isoenzymes might be involved. Indeed, in this study we show that a large increase in the amount of rat heart PDK4 protein correlates with the stable increase in PDK activity that occurs in the hearts of starved and diabetic rats. That the increase in PDK4 protein may account in large part if not entirely for the increase in PDK activity is suggested by the magnitude of the PDK isoenzyme shift induced by starvation and diabetes. Quantitative Western-blot data indicated that PDK4 increased from less than one-third of the total PDK protein to more than 50 % of the total in the starved state and to 75 % of the total in the diabetic state. These findings, coupled with our finding that the specific enzyme activity of recombinant PDK4 is nearly 7 times greater than recombinant PDK2 [21], indicate that the isoenzyme shift caused primarily by changes in amount of PDK4 must have a large impact on total PDK activity. Thus this study provides the first molecular explanation for the stable increase in PDK activity that occurs in rat heart under the metabolic conditions that prevail in starved and diabetic animals. As a result of these findings we hypothesize that an increase in PDK activity caused by an increase in the amount of PDK4 protein leads to greater phosphorylation and lower activity of the pyruvate dehydrogenase complex. This suppresses glucose and lactate oxidation but favours fatty acid oxidation in the hearts of starved and diabetic animals. Since stable changes in PDK activity occur in several other tissues [10,12,13,15], the likelihood is that shifts in the composition of the PDK isoenzymes will be found to be a universal mechanism throughout the body for regulation of PDK activity and therefore the activity state of the pyruvate dehydrogenase complex.

An increase in the relative contributions of PDK4 to the total PDK activity of rat heart mitochondria can explain several key observations reported in previous studies of starved and diabetic animals. For example, inhibition of the synthesis of PDK4 is now the simplest explanation for why the protein synthesis inhibitor cycloheximide blocks the increase in rat heart PDK activity that normally occurs during starvation [10]. Likewise, the underlying cause of the latency of re-activation of the pyruvate dehydrogenase complex upon re-feeding of starved animals [10,29], suggested previously to be physiologically important for mobilization of accumulated triacylglycerol in the heart [3,30], probably reflects time required to down-regulate the amount of PDK4. Finally, starvation and diabetes have been shown to induce a decrease in the sensitivity of heart PDK to inhibition by dichloroacetate [16,23,31]. The isoenzyme shift observed in the present study offers an explanation for this loss of sensitivity, since PDK4 is less sensitive to dichloroacetate inhibition than PDK2 [21].

Previous work from Randle's laboratory [14,32] has established that starvation induces a significant increase (4.5-fold) in the specific activity (units/mg of protein) of PDK purified from rat liver. A small increase in PDK protein that could account for no more than about 10 % of the increase in PDK activity was found to occur in starved liver. It was proposed that starvation might increase the specific activity of PDK in liver by either covalent modification or an increase in the concentration of a more active isoform [32]. Although liver was not studied in the present work, our findings are consistent with the operation of the latter mechanism in the heart.

The levels of three of the four PDK isoenzymes known to exist in mammalian tissues were measured in this study. PDK3 protein

levels were not determined for want of antibodies specific for PDK3 protein. Probing multiple rat tissue Northern blots with a human PDK3 cDNA has shown that a rat homologue of human PDK3 probably exists [21]. However, the message for this particular isoenzyme is expressed in greatest abundance in testes, with apparently quite low amounts in rat heart relative to the messages for the other PDK isoenzymes [21]. These findings make it unlikely, but do not exclude the possibility, that an increase in amount of PDK3 is also a factor in the stable increase in PDK activity that occurs in starvation and diabetes.

The gene encoding human PDK4 was originally identified by positional cloning in a region on chromosome 7q linked with non-insulin-dependent diabetes mellitus in Pima Indians [20]. Alterations in this gene have been ruled out as a likely molecular explanation for linkage of this region with this type of diabetes [20]. Nevertheless, previous studies have shown that insulin decreases PDK activity in skeletal muscle of diabetic animals [15] and also effectively prevents apparent induction of PDK activity by 3,5,3'-tri-iodothyronine, octanoate and dibutyl cAMP in cultured cardiac myocytes [33]. These results agree well with the finding in this study that insulin treatment of diabetic rats reduced PDK activity and lowered the level of PDK4 protein in heart mitochondria. It seems likely therefore that a mechanism exists by which insulin decreases the amount of PDK4. Elevated PDK activity as the result of increased amounts of PDK4 in the diabetic state, regardless of whether induced by insulin insufficiency or insulin resistance, would be expected to make the pyruvate dehydrogenase complex resistant to dephosphorylation and therefore activation by pyruvate dehydrogenase phosphatase. This in turn could exacerbate the impaired glucose oxidation and insulin resistance characteristic of non-insulin-dependent diabetes mellitus.

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