Starvation and Diabetes Reduce the Amount of Pyruvate Dehydrogenase Phosphatase in Rat Heart and Kidney

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The pyruvate dehydrogenase complex (PDC) is inactivated in many tissues during starvation and diabetes to conserve three-carbon compounds for gluconeogenesis. This is achieved by an increase in the extent of PDC phosphorylation caused in part by increased pyruvate dehydrogenase kinase (PDK) activity due to increased PDK expression. This study examined whether altered pyruvate dehydrogenase phosphatase (PDP) expression also contributes to changes in the phosphorylation state of PDC during starvation and diabetes. Of the two PDP isoforms expressed in mammalian tissues, the Ca^{2+}-sensitive isoform (PDP1) is highly expressed in rat heart, brain, and testis and is detectable but less abundant in rat muscle, lung, kidney, liver, and spleen. The Ca^{2+}-insensitive isoform (PDP2) is abundant in rat kidney, liver, heart, and brain and is detectable in spleen and lung. Starvation and streptozotocin-induced diabetes cause decreases in PDP2 mRNA abundance, PDP2 protein amount, and PDP activity in rat heart and kidney. Refeeding and insulin treatment effectively reversed these effects of starvation and diabetes, respectively. These findings indicate that opposite changes in expression of specific PDK and PDP isoenzymes contribute to hyperphosphorylation and therefore inactivation of the PDC in heart and kidney during starvation and diabetes. *Diabetes* 52:1371–1376, 2003

The pyruvate dehydrogenase complex (PDC) catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA, a reaction that links the utilization of glycogen, glucose, and lactate with the citric acid cycle to meet the energy needs of cells. The activity of the PDC is tightly controlled by a phosphorylation/dephosphorylation cycle, with the extent of phosphorylation of the complex determined by the relative activities of the pyruvate dehydrogenase kinase (PDK) versus the pyruvate dehydrogenase phosphatase (PDP). Induced changes in PDK activity relative to PDP activity during transitions to different nutritional and hormonal states lead to substantial changes in the phosphorylation state and therefore the activity state of PDC. It is now appreciated that altered expression of PDK4, one of four isoenzymes of PDK expressed in mammalian tissues, plays a major role in determining the activity state of PDC. Starvation (1–5) and diabetes (2,3) cause an increase in PDK4 expression in various tissues. Refeeding of starved rats (2–5) and insulin treatment of type 1 diabetic rats (2,3) effectively downregulate PDK4 expression. Whether PDP expression is also affected by the nutritional and hormonal state of animals has received less attention. It has been reported, however, that starvation decreases PDP activity in rat mammary gland and kidney (6,7), raising the possibility that changes in PDP expression opposite to those of PDK may be induced by starvation in order to maximize the extent of PDC phosphorylation in this nutritional state.

Mammalian tissues contain two genetically and biochemically different forms of PDP designated as PDP1 and PDP2 (8). Both are Mg^{2+}-dependent mitochondrial serine/threonine phosphoprotein phosphatases belonging to the protein phosphatase 2C family (8,9). The Ca^{2+}-sensitive isoform, PDP1, has long been recognized as an important regulator of the activity state of the PDC (9). Its sensitivity to Ca^{2+} leads to activation of the PDC in response to conditions that increase the mitochondrial concentrations of this cation (rev. in 10). The more recently identified PDP2 is insensitive to stimulation by Ca^{2+} (8). Its K_m for Mg^{2+} is higher than that of PDP1, and its activity is stimulated by spermine (8).

The purpose of the present study was to determine whether mechanisms exist for long-term regulation of PDP expression in mammalian tissues. Here we report the finding that starvation and chemical-induced diabetes, conditions leading to hyperphosphorylation and downregulation of PDC activity, are associated with stable changes in PDP2 expression in rat heart and kidney.

RESEARCH DESIGN AND METHODS

Materials. Male Wistar rats were from Harlan Industries (Indianapolis, IN). Protamine-zinc insulin for the treatment of diabetic rats was from Anthony Products (Arcadia, CA). Ultraspec RNA isolation system was from Biotecx Laboratories (Houston, TX). [γ-^{32}P]ATP, [α-^{32}P]dCTP, and ^{125}I-Protein A were obtained from NEN Life Science Products (Boston, MA). High prime DNA labeling kit was from Roche Applied Science (Indianapolis, IN). QuickHyb hybridization solution and sonicated salmon sperm DNA were from Stratagene (La Jolla, CA). Rat multiple tissue Northern blot and Chroma spin + TE10 column were purchased from Clontech (Palo Alto, CA). Phosphatase inhibitor cocktail set I was from Calbiochem (La Jolla, CA). Other chemicals were from Sigma (St. Louis, MO). The Biochemistry Biotechnology Facility (Indianapolis, IN) synthesized and purified the phosphorylated peptide used in the phosphatase assay.

Animals. Male Wistar rats weighing ~200 g were maintained in a temperature- and light-controlled animal room and fed with rodent laboratory chow ad

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E1α, α subunit of pyruvate dehydrogenase component of pyruvate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase.

libitum from Purina Mills (Richman, IN) for at least 3 days before initiating the experiments. To determine the effect of starvation and refeeding, food but not water was withheld from a group of rats for 48 h. Half of the rats were killed and the other half refed with chow diet for another 48 h before being killed. A separate control group of rats was fed the chow diet throughout the time period. To determine the effect of diabetes, overnight-fasted rats were injected intravenously with streptozotocin (65 mg/kg body wt) dissolved in 0.05 mol/l sodium citrate, pH 4.5. Free access to food and water was then provided during the experimental period. Control rats and half of the diabetic rats were killed 48 h after streptozotocin injection. The remaining diabetic rats received subcutaneous injection of protamine-zinc insulin (1.2 units/100 g body wt) every 12 h until they were killed 48 h later. The last insulin injection was given 2 h before they were killed. Blood glucose levels were monitored to verify induction of diabetes and the effectiveness of the insulin treatments. Half of the amount of each tissue sample removed from the rats was freeze clamped at the temperature of liquid nitrogen for RNA isolation. The other half was chilled on ice for isolation of mitochondria.

Western blot analysis. Mitochondria from rat heart, kidney, and liver were isolated as described elsewhere (11). Mitochondrial proteins were separated on 10% SDS-PAGE and blotted onto nitrocellulose membrane (15 μg protein/line). Blots were probed with polyclonal antisera raised against recombinant rat PDP1 and PDP2. Antiserum against native PDC was used to quantitate PDC E1α (α subunit of pyruvate dehydrogenase component of PDC) as a loading control. Immunoreactive bands were visualized as previously described (12).

Northern blot analysis. Total RNA was extracted from freeze-clamped tissues with Ultraspec RNA isolation system following the instructions of the manufacturer. Fifteen micrograms of total RNA from each sample were used for Northern blotting analysis, conducted as previously described (13). The tissue distribution of PDP isoenzymes was investigated using Northern blot analysis with rat multiple tissue Northern blot (Clontech). PDP1 and PDP2 mRNA were detected with corresponding ^{32}P-labeled cDNA probes.

Image quantification and data analysis. Relative intensities of bands obtained in Western and Northern blot analysis were quantified with UN-SCAN-IT Software, Version 4.1, from Silk Scientific (Orem, UT). SigmaPlot, Version 3.02, from SPSS (Chicago, IL) was used for data analysis.

PDP assay. PDP activity was determined by the release of inorganic phosphate from a phosphorylated PDC peptide with the Nonradioactive Phosphatase Assay System from Promega (Madison, WI) as described by Caruso et al. (14) with some modifications. The polypeptide substrate [Ac-Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser(P)-Tyr-Arg-NH_2] was synthesized according to the amino acid sequence surrounding phosphorylation sites 1 and 2 of the PDC E1α component (15). Isolated mitochondria were suspended in a buffer containing 50 mmol/l Tris-HCl (pH 7.4), 2 mmol/l dithiothreitol, 2% bovine serum, 0.05% (v/v) Triton X-100, and 0.05 mmol/l EGTA. Suspensions were frozen and thawed three times before centrifugation at 100,000 rpm for 80 min in a Beckman TL-100 ultracentrifuge to pellet insoluble material including the PDC. Supernatants were collected and desalted with Chroma spin + TE10 columns to remove endogenous inorganic phosphate. Phosphatase reactions were initiated by adding 30 μg of soluble mitochondrial protein into the 50-μl reaction mixture containing 50 μmol/l substrate peptide, 20 mmol/l imidazole buffer (pH 7.2), 10 mmol/l $MgCl_2$, 0.1 mmol/l EGTA, 0.1% β-mercaptoethanol, and 1 mg/ml BSA. Reaction cocktails were supplemented with phosphatase inhibitor cocktail set I [25 μmol/l (-)-*p*-bromotetramisole oxalate, 5 μmol/l cantharidin, and 5 nmol/l microcystin-LR] in order to suppress the activities of PP1 and PP2A phosphatases that may contaminate the mitochondrial extracts. After 30 min of incubation at 30°C, the reactions were terminated with Molybdate Dye Solution (Promega, Madison, WI). PDP activity is expressed as nmoles of phosphate released per minute per milligram of mitochondrial extract protein.

RESULTS

Tissue distribution of PDP1 and PDP2 messages in the rat. By Northern blot analysis, PDP1 mRNA was present in all tissues examined but was expressed most abundantly in heart, brain, and testis (Fig. 1).

PDP1 was also the predominant form in skeletal muscle, even though the basal level of PDP1 expression was low in this tissue relative to the levels present in heart, brain, and testis. In contrast, mRNA for PDP2 was very abundant in heart, brain, liver, and kidney (Fig. 1). PDP2 message was also present in rat spleen and lung but was below the level of detection in rat skeletal muscle and testis. These findings provide evidence for different patterns of PDP1 and PDP2 expression in tissues, which may allow for tissue-specific regulation of PDC activity in different physiological states. Interestingly, two bands corresponding to PDP2 mRNA were detected in all tissues examined. Two bands for PDP1 were likewise detected in brain and to a much lesser extent in heart. Alternative splicing may be responsible for this heterogeneity but has not been investigated.

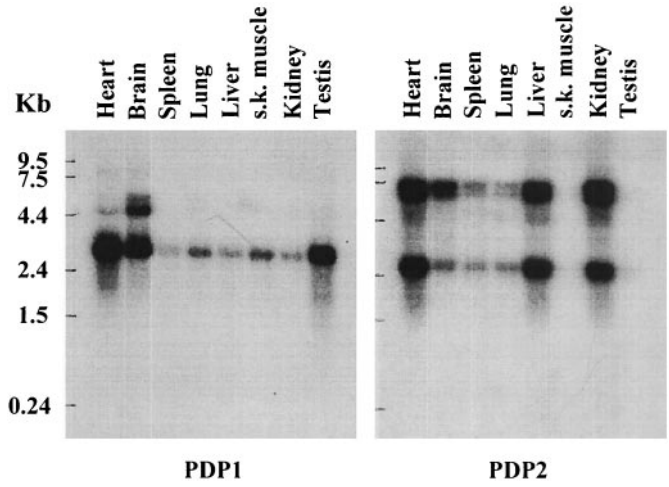

FIG. 1. Relative abundance of PDP1 and PDP2 mRNAs in rat tissues. Rat multiple tissue Northern blot (2 μg poly A RNA from each tissue) was hybridized with PDP1 and PDP2 cDNA probes, respectively. Exposure time of Northern blots to X-ray film was 10 h for PDP1 and 15 h for PDP2.

Effect of starvation on relative abundance of PDP1 and PDP2 protein and mRNA in rat heart and kidney. Starvation for 48 h induced significant decreases in the amounts of PDP2 protein expressed in the heart (1.00 ± 0.09 in well-fed rats vs. 0.25 ± 0.03 in starved rats, $n = 4$; $P < 0.01$) and the kidney (1.00 ± 0.04 in well-fed rats vs. 0.45 ± 0.09 in starved rats, $n = 4$; $P < 0.01$) (Figs. 2*A* and 3*A*).

Corresponding decreases in PDP2 message abundance occurred in the heart (1.00 ± 0.07 in well-fed rats vs. 0.41 ± 0.01 in starved rats, $n = 4$; $P < 0.01$) and the kidney (1.00 ± 0.09 in well-fed rats vs. 0.57 ± 0.07 in starved rats, $n = 4$; $P < 0.01$) (Fig. 2*B* and 3*B*). Refeeding of starved rats for 48 h resulted in nearly complete recoveries of PDP2 protein and message in both tissues (Figs. 2 and 3).

Although PDP1 is expressed in relatively large amounts in the heart, starvation for 48 h had no effect on the protein and message levels of this phosphatase in rat heart (Fig. 2). In the kidney, where expression of PDP1 is very low relative to PDP2, starvation induced a further decrease in PDP1 protein (1.00 ± 0.05 in well-fed rats vs. 0.58 ± 0.13 in starved rats, $n = 4$; $P < 0.01$; Fig. 3*A*) but had little if any effect on PDP1 message (Fig. 3*B*). Refeeding partially reversed the effect of starvation on the level of PDP1 protein (Figs. 3*A*).

Starvation of rats for 48 h was required to observe the effects shown in Figs. 2 and 3 on PDP2 message and protein in kidney and heart. Starvation for 24 h did not induce significant changes in the PDP2 expression in these tissues.

Effect of streptozotocin-induced diabetes on PDP2 protein amount and mRNA abundance in rat heart and kidney. Induction of diabetes by streptozotocin was

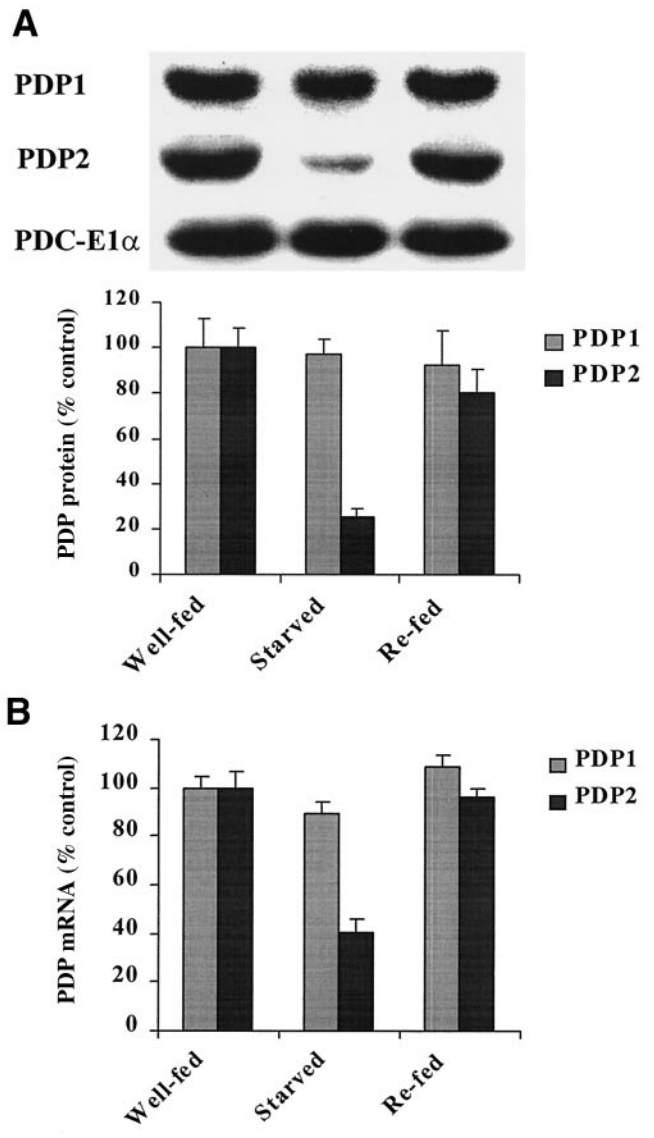

FIG. 2. Effects of starvation and refeeding on PDP1 and PDP2 protein and mRNA amounts in rat heart. *A*: *Upper panel*: Autoradiograph of representative Western blot of PDP1 and PDP2 protein in rat heart mitochondria. Lanes correspond to 15 μg total mitochondrial protein. *Lower panel*: Quantitative changes of relative abundance of PDP1 and PDP2 protein in rat heart mitochondria. *B*: Quantitative changes of relative abundance of PDP1 and PDP2 message in 15 μg total RNA prepared from rat heart as determined by Northern blot analysis. Data points represent means ± SE of four rats in each group.

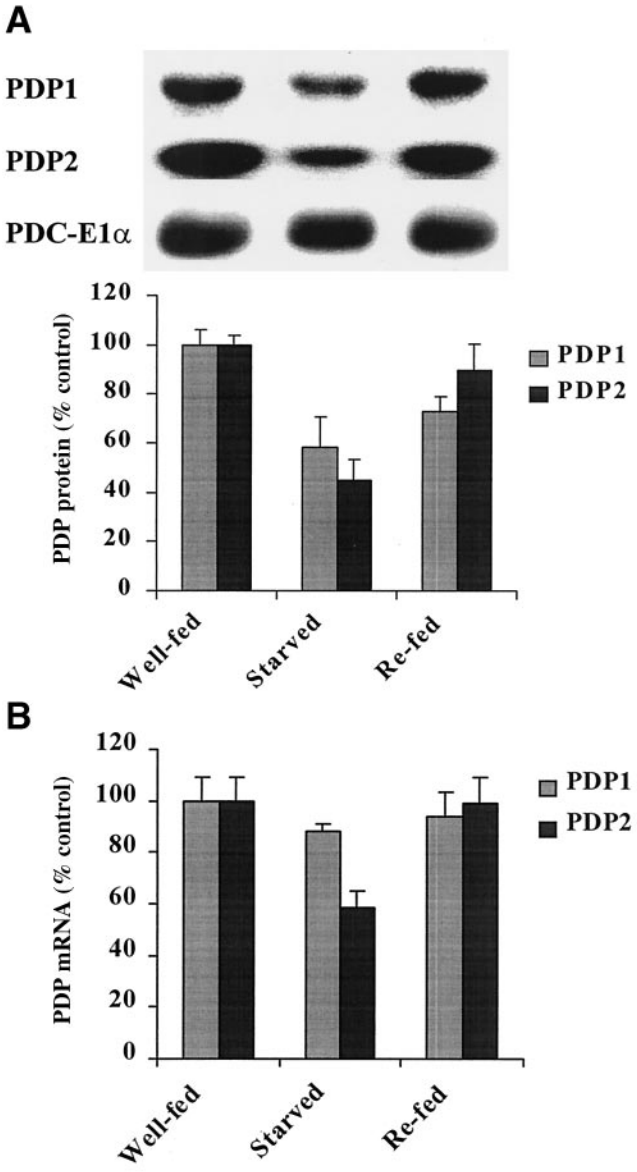

FIG. 3. Effects of starvation and refeeding on PDP1 and PDP2 protein and mRNA amounts in rat kidney. *A*: *Upper panel*: Autoradiograph of representative Western blot of PDP1 and PDP2 protein in rat kidney mitochondria. Lanes correspond to 15 μg total mitochondrial protein. *Lower panel*: Quantitative changes of relative amount of PDP1 and PDP2 protein in rat kidney mitochondria. *B*: Quantitative changes of relative abundance of PDP1 and PDP2 message in 15 μg total RNA prepared from rat kidney as determined by Northern blot analysis. Data points represent means ± SE of four rats in each group.

confirmed by the measurement of blood glucose levels (390 ± 25 mg/dl at 48 h after administration of the drug vs. 95 ± 8 mg/dl in controls). Treatment of the diabetic rats with long-lasting insulin at 12-h intervals for 48 h was effective in lowering blood glucose levels to the normal control range (<100 mg/dl). Diabetes induced by streptozotocin caused substantial decreases in the levels of PDP2 protein in heart (1.00 ± 0.03 in control rats vs. 0.33 ± 0.04 in diabetic rats, $n = 6$; $P < 0.01$) and kidney (1.00 ± 0.05 in control rats vs. 0.63 ± 0.07 in diabetic rats, $n = 6$; $P < 0.01$) (Fig. 4*A*).

Corresponding decreases in PDP2 mRNA levels occurred in the heart (1.00 ± 0.07 in control rats vs. 0.37 ± 0.04 in diabetic rats, $n = 6$; $P < 0.01$) and the kidney (1.00 ± 0.07 in the control rats vs. 0.66 ± 0.04 in the diabetic rats, $n = 6$; $P < 0.01$) (Fig. 4*B*). These effects were completely reversed by insulin treatment (Fig. 4*A* and *B*, respectively). Levels of PDP1 protein in these tissues remained relatively constant under these experimental conditions (data not shown).

Effect of starvation and diabetes on PDP activity in mitochondria isolated from rat heart and kidney. Starvation induced modest but significant decreases in PDP activity in both heart (18%) and kidney (32%) (Table 1).

Refeeding the animals induced complete restoration of PDP activity in both tissues (Table 1). Diabetes caused 26 and 28% reductions in PDP activity in heart and kidney,

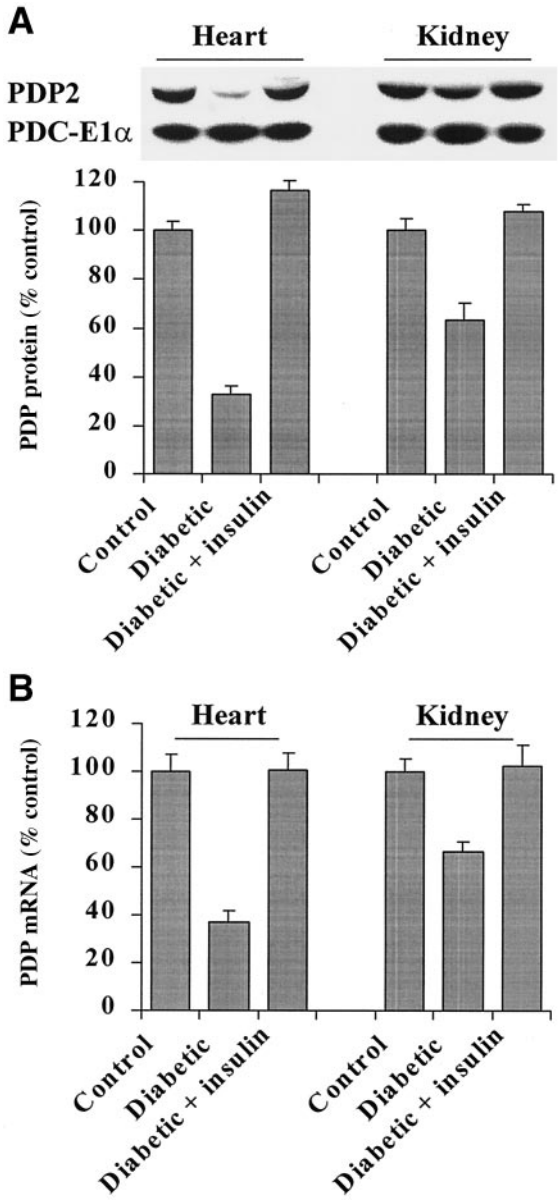

FIG. 4. Effects of diabetes and insulin treatment on PDP2 protein and mRNA amounts in rat heart and kidney. *A*: Quantitative changes of relative amount of PDP2 protein in 15 μg total mitochondrial protein prepared from the heart and kidney of control, diabetic, and insulin-treated diabetic animals as determined by Western blot analysis. *B*: Quantitative changes of relative abundance of PDP2 message in 15 μg total RNA prepared from the heart and kidney of control, diabetic, and insulin-treated diabetic animals as determined by Northern blot analysis. Data points represent means ± SE of six rats in each group.

respectively. Insulin treatment partially restored PDP activity in both tissues (Table 1).

TABLE 1
Effects of starvation and diabetes on PDP activity in rat heart and kidney mitochondria

	PDP activity (nmol · min^{-1} · mg protein^{-1})	
Animal treatment	Heart	Kidney
Well-fed	1.22 ± 0.04	1.02 ± 0.05
Starved (48 h)	1.00 ± 0.06*	0.68 ± 0.03†
Starved-refed (48 h)	1.20 ± 0.11	1.00 ± 0.05
Control	1.54 ± 0.05	1.70 ± 0.08
Diabetic	1.14 ± 0.04‡	1.22 ± 0.08‡
Insulin-treated diabetic	1.40 ± 0.01	1.45 ± 0.09

Data are means ± SE for tissue samples obtained from 4–6 animals in each group; *$P < 0.05$ compared with well-fed group; †$P < 0.01$ compared with well-fed group; ‡$P < 0.01$ compared with control group.

DISCUSSION

Survival during starvation is dependent upon mechanisms that limit oxidative loss of pyruvate in nonneuronal tissues of the body. Pyruvate and other three-carbon compounds that can be converted to glucose must be conserved during starvation. Efficient recycling of these compounds is particularly important in limiting utilization of glucogenic amino acids that otherwise would have to be used to maintain glucose levels during starvation. Limiting amino acid turnover helps keep proteolysis in check and conserves body protein (16,17). Inhibition of pyruvate oxidation is achieved by a combination of regulatory mechanisms that virtually eliminate nonneuronal cell pyruvate dehydrogenase activity during starvation. Most important among these is covalent modification of the complex by phosphorylation. The balance between the relative activities of the PDKs and pPDPs determines the extent to which the complex is inhibited by phosphorylation. It is well established that increased PDK expression in starvation shifts the balance toward greater phosphorylation of the complex. Whether stable changes in PDP activity also occur is much less certain. Some of the increase in PDK activity induced by starvation is due to elevated concentrations of free fatty acids and ketone bodies. Catabolism of these compounds increases the levels of NADH and acetyl-CoA (18,19), which in turn increase PDK activity by reduction and acetylation of the lipoyl residues of the E2 component of the complex (20,21). Complementing this mechanism is a marked increase in PDK4 expression in many tissues (1–5), along with some increase in PDK2 expression in a few tissues (3,5). Changes in blood levels of glucocorticoids, insulin, and free fatty acids signal increased PDK4 expression in starvation (22).

Although a role for greater PDK activity in promoting PDC phosphorylation during starvation is well established and appropriate in this physiological state (1–5), it is obvious that a decrease in PDP activity could induce the same effect. Because Ca^{2+} stimulates PDP1 activity (9), factors that lower mitochondrial calcium levels decrease phosphatase activity. Although higher Ca^{2+} levels are likely involved in activation of the PDC in heart by epinephrine and increased work load (rev. in 10), evidence is lacking that would implicate changes in Ca^{2+} concentration in regulation of PDP activity during starvation. On the other hand, rapid activation of the PDC by insulin, purportedly by activation of PDP, has been reported in many studies (rev. in 23). Because insulin levels are decreased in starvation, it follows that PDP activity could be decreased for want of insulin. Indeed, recent evidence suggests a role for stimulation of protein kinase Cδ in insulin-mediated PDP activation (14,24). However, the activating effect of insulin on the PDC lasts only a few minutes (14,24), making it uncertain whether the decrease in insulin during starvation would have a lasting effect upon the activity state of the complex by this mechanism.

The possibility that stable changes in PDP activity could be induced has been examined in the past with mixed results. The recent finding that two isoforms of this enzyme are expressed in a tissue-specific manner, one sensitive and one insensitive to Ca^{2+} stimulation (8), revealed an unexpected level of complexity that might help to explain why changes in PDP activity have been seen in response to starvation in some studies but not in others. The present study provides additional information about tissue specificity of PDP isoform expression. The liver and the kidney express PDP2 and almost no PDP1, the testes and skeletal muscle express PDP1 but perhaps no PDP2, and the heart and the brain express both PDP1 and PDP2. Assuming that protein levels of the PDPs correspond to message levels, Ca^{2+} activation of PDP1 activity is likely important in the heart, skeletal muscle, brain, and testes, whereas Ca^{2+}-independent mechanisms for regulation of PDP2 activity are likely present in the liver and kidney and perhaps the heart and brain. Failures to detect effects of different metabolic states on phosphatase activity in heart and adipose tissue have been reported (19,25,26). In contrast, clear evidence for decreased PDP activity in rat kidney and lactating mammary gland in response to starvation has been reported (6,7,27). The present study confirms that a decrease in kidney PDP activity is induced by starvation and also provides evidence that this can be due, at least in part, to decreased expression of the PDP2 protein. A modest decrease in PDP activity in response to starvation was also found with rat heart, consistent with our finding of a significant decrease in the level of PDP2 expression in this tissue.

Diabetes caused by chemical destruction of pancreatic β-cells is also known to cause hyperphosphorylation and therefore inactivation of the pyruvate dehydrogenase complex in many tissues of the rat (rev. in 28). As in starvation, increased PDK activity (29,30) occurs that can be explained at least in part by increased PDK4 expression (1) as a consequence of increased levels of free fatty acids and glucocorticoids and decreased levels of insulin (22). As would be predicted from the findings with starvation, diabetes also resulted in decreased PDP activity and decreased PDP2 expression in kidney and heart. From these findings it appears likely that concurrent upregulation of PDK expression and downregulation of PDP expression contribute to hyperphosphorylation of PDC in diabetes. This, of course, conserves three-carbon compounds for hepatic gluconeogenesis and contributes to the hyperglycemia characteristic of diabetes.

Streptozotocin is a cytotoxic molecule that induces diabetes by destroying pancreatic β-cells. Although the action of streptozotocin is quite selective for β-cells, cytotoxic effects do occur in other tissues (31), raising the possibility that streptozotocin-induced decrease in PDP2 expression may be due to a direct toxic effect rather than the diabetic state. However, starvation, which like diabetes lowers insulin levels, caused the same effects on PDP2 expression as streptozotocin, and the effects of starvation were reversed completely by refeeding, which increases insulin levels. Moreover, treatment of rats with streptozotocin with the same conditions used in this study causes the opposite change in PDK4 expression, i.e., causes a marked increased in its level (1,2). And most important, treating steptozotocin-diabetic rats with insulin completely restored normal levels of PDP2 (this study) and PDK4 (1,2). These findings strongly suggest that the changes in the expression of the PDC regulatory enzymes are a result of the diabetic state rather than a general toxic effect of streptozotocin.

In general, changes in PDP activity followed changes in PDP2 mRNA and protein. However, the overall decrease of total PDP activity in rat heart in response to starvation and diabetes was smaller than the decrease of PDP2 protein and message. This may reflect background phosphatase activity caused by the presence of other phosphoprotein phosphatases in the mitochondrial extracts. Although a cocktail of phosphatase inhibitors was included in the phosphatase assay to minimize the contribution of alkaline phosphatase and other serine/threonine phosphatase, it may not be possible to suppress the activities of the contaminating phosphatase without inhibiting PDP2 activity. Other members of the protein phosphatase 2C family may also provide background activities to PDP, particularly since they are capable of dephosphorylating PDC (rev. in 32). PDP1 is very abundant in rat heart, but without change under starvation and diabetes it may also contribute to the background activity. The observed decrease in PDP activity in rat kidney was closer to the observed decrease in PDP2 protein, consistent with the observation that PDP2 is the dominant PDP isoenzyme expressed in this tissue.

The finding that insulin treatment efficiently reverses the effect of diabetes on PDP2 protein and message suggests that insulin may be involved in long-term regulation of its expression. Regulation at the level of either transcription or message stability probably accounts for the reasonably good agreement between changes in message and protein levels.

No attempt was made to examine PDP levels and activity in the different cell types and mitochondrial subpopulations that exist in heart and kidney. This is of interest and will be pursued in future studies. Significant differences are known to exist in regulation of PDK expression in different muscle fiber types (5,33).

In conclusion, this study demonstrates that starvation and diabetes decrease PDP2 expression in rat kidney, a tissue in which PDP2 is the dominantly expressed phosphatase, and rat heart, a tissue that also expresses large amounts of PDP1. These findings suggest that opposite changes in PDP2 and PDK4 expression contribute to the hyperphosphorylation and inhibition of PDC activity in these tissues by starvation and diabetes. Whether regulation of PDP expression is an important mechanism in other tissues remains to be established.

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