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Metabolism Clinical and Experimental 55 (2006) 317–323

Metabolism
Clinical and Experimental

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Increased expression of hepatic pyruvate dehydrogenase kinases 2 and 4 in young and middle-aged Otsuka Long-Evans Tokushima Fatty rats: induction by elevated levels of free fatty acids

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Received 5 February 2005; accepted 7 September 2005

Abstract

The activity of the pyruvate dehydrogenase complex (PDC) is regulated by covalent modification of its E1 component, which is catalyzed by specific pyruvate dehydrogenase kinases (PDKs) and phosphatases. In the liver, PDK2 and PDK4 are the most abundant PDK isoforms, which are responsible for inactivation of PDC when glucose availability is scarce in the body. In the present study, regulatory mechanisms of hepatic PDC were examined before and after the onset of type 2 diabetes mellitus in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, using Long-Evans Tokushima Otsuka (LETO) rats as controls. Plasma glucose and insulin concentrations were at normal levels in rats aged 8 weeks, but were significantly higher in OLETF than in LETO rats aged 25 weeks, indicating insulin resistance in OLETF rats. Plasma free fatty acids (FFAs) were 1.6-fold concentrated, and the liver PDC activity was significantly lower in OLETF than in LETO rats at both ages, suggesting suppression of pyruvate oxidative decarboxylation in OLETF rats before and after the onset of diabetes. Pyruvate dehydrogenase kinase activity and abundance of PDK2 and PDK4 proteins, as well as mRNAs, were greater in OLETF rats at both ages. These results suggest that persistently elevated levels of circulating free fatty acid in normal and diabetic OLETF rats play an important role in stimulating PDK2 and PDK4 expression in liver.
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1. Introduction

Mammalian pyruvate dehydrogenase complex (PDC), located in the matrix of mitochondria, catalyzes the oxidative decarboxylation of pyruvate with formation of carbon dioxide, acetyl-coenzyme A (CoA), and NADH. This reaction is irreversible and links glycolysis and the citric acid cycle in mitochondria [1]. Thus, because of generation of acetyl-CoA, the relative activity of PDC plays an important role in dictating the rate at which carbohydrate-derived substrates will be fully oxidized in tissues with large energy demands such as brain, muscle, and heart, or biotransformed into fatty acids and cholesterol in lipogenic tissues such as adipose, mammary gland, and liver [2].

The activity of PDC is controlled by an intricately regulated cycle carried out by dedicated kinase and phosphatase components [3]. Inactivation of the complex is catalyzed by the pyruvate dehydrogenase kinase (PDK), which phosphorylates the serine residues of the E1α subunit of PDC [4]. In contrast, pyruvate dehydrogenase phosphatase reactivates the complex by dephosphorylating E1α [1]. Kinase and phosphatase relative activities depend upon intramitochondrial concentrations of different metabolites and cofactors [5] that are, in turn, dependent upon nutritional and hormonal states. Long-term regulation of PDC, instead, occurs by differential profusion of these covalent regulators, particularly PDK, which tightly binds to the multienzyme complex.

Among the 4 known eukaryotic PDK isoenzymes, PDK2 and PDK4 are the most abundant isoforms in liver [6] and are responsible for inhibition of PDC in this organ during

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0026-0495/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2005.09.014

starvation [7,8] or triiodothyronine-induced hyperthyroidism [7]. In other tissues such as skeletal muscle, PDK4 has been shown to be predominantly overexpressed in starved [9-11], streptozotocin (STZ)–induced diabetic [11], and high fat–fed rats [12]. Furthermore, treatment of rats with a relatively specific ligand for the peroxisome proliferator–activated receptor α (PPARα), WY-14,643, has been reported to mimic the effects of starvation and chemically induced diabetes on PDK4 expression in skeletal muscle [11] and liver [13].

Provided that hyperthyroidism, starvation, and high-fat feeding significantly increase circulating free fatty acids (FFAs), which function as endogenous PPARα agonists [14,15], we hypothesize that, during these conditions, elevation in FFA concentrations might play an essential role in the mechanisms that up-regulate PDK4, with consequent inhibition of PDC activity. Moreover, although the working factors responsible for regulation of the PDK isoform expression during physiological conditions such as obesity-related insulin resistance and type 2 diabetes mellitus remain to be identified, high plasma FFA levels may possibly underlie transcriptional increases of these genes.

In the present study, as a continuation of our previous research [16], the type 2 diabetes mellitus model Otsuka Long-Evans Tokushima Fatty (OLETF) rat was used to investigate the mechanisms that regulate PDC activity in hepatic tissue because the liver plays an important role with regard to maintenance of glucose homeostasis of the body under particular metabolic conditions and also because the regulation of PDC activity by specific kinases before and after the onset of type 2 diabetes mellitus is, to our knowledge, an unknown issue for hepatic tissue. Adult OLETF rats have hyperglycemic obesity with hyperinsulinemia and insulin resistance identical to that of human type 2 diabetes mellitus [17]. Male animals own innate polyphagia with rapid body weight gain and accumulation of intra-abdominal fats, leading to development of hyperlipidemia and insulin resistance from 12 weeks of age [17,18]. In addition, sedentary young [19] and middle-aged [20] OLETF rats were reported to exhibit significantly higher levels of circulating FFA than their nondiabetic/lean counterparts, Long-Evans Tokushima Otsuka (LETO) rats. Therefore, we speculate that PPARα activation by elevated FFA levels in OLETF rats may signal up-regulation of PDKs in liver, leading to sparing of 3-carbon compounds, not only during the diabetic state, but also before the onset of diabetes mellitus.

The aim of the present study therefore was to examine concomitantly (1) the mechanisms by which PDC activity is regulated in both young/normal and middle-aged/diabetic OLETF rats and (2) the relation between plasma FFA concentrations and expression of hepatic PDKs. By investigating these regulatory pathways, the present study sheds new light on the factors that control hepatic metabolism in vivo associated with development of type 2 diabetes mellitus.

2. Materials and methods

2.1. Materials

Polyclonal antisera against specific recombinant rat PDK2 [21] and PDK4 [6] were generously provided by Professor Robert A. Harris (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN). These antibodies have been tested for cross-reactivity [22] and used by others to detect PDK2 [9] and PDK4 [7] proteins in rat tissues. Arylamine acetyltransferase was partially purified from pigeon liver acetone powder (Sigma, St Louis, MO) using the procedures described by Brooks and Storey [23], as depicted in our previous report [16]. Expression and purification of recombinant pyruvate dehydrogenase phosphatase 1 were performed by the method of Huang et al [5]. Enhanced chemiluminescence (ECL) reagents and ^{32}P nucleotides were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Bradford reagent for protein estimation and goat antirabbit secondary antibody were purchased from Bio-Rad Laboratories (Hercules, CA). Polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rabbit antigoat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and biochemicals were of analytical grade and were purchased from Wako (Osaka, Japan), Oriental Yeast (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich (Tokyo, Japan).

2.2. Animals

All procedures involving LETO and OLETF rats were conducted as previously described [16]. Ten female Sprague-Dawley (SD) rats aged 10 weeks (body weight, 226 ± 4 g) were used to investigate the effects of artificial elevation of FFA concentrations on the abundances of hepatic PDK2 and PDK4. After a 5-hour fasting period, experimental animals (n = 5) were intragastrically administered with 3.8 mL corn oil, followed by subcutaneous injection of 0.3 mL heparin (300 U) 1 hour later; at the same time, control animals (n = 5) were treated with equal amounts of 1% (wt/vol) carboxymethyl cellulose and 0.9% (wt/vol) NaCl, respectively [24]. Sprague-Dawley rats were euthanized as described above for LETO and OLETF rats 4.5 hours after injection of heparin or saline.

2.3. Blood and tissue sampling and analytical methods

Sampling procedures and plasma and tissue preservation conditions until analyses were described previously [16]. Assays of the plasma parameters were carried out as described [16], and the liver glycogen concentrations were measured using the method of Lo et al [25].

2.4. Enzyme assays

The method used for assaying hepatic activated PDC activity (ie, the activity in vivo; complex partially in active/

dephosphorylated state) and hepatic total PDC activity (ie, activity of the fully active/dephosphorylated enzyme) was described elsewhere [16].

Pyruvate dehydrogenase kinase activities of liver tissue extracts were measured by evaluating the rate at which PDC is phosphorylated/deactivated by endogenous PDK upon incubation with ATP [16]. The buffer used for PDK reaction contained 20 mmol/L HEPES (pH 7.4 with KOH), 20% (wt/vol) glycerol, 1.5 mmol/L $MgCl_2$, and 5 mmol/L 2-mercaptoethanol.

2.5. Electrophoresis and immunoblotting analysis

Procedures for detecting and quantifying the amounts of hepatic PDK2 and PDK4 proteins were carried out as described elsewhere [16]. In addition, with the aim of adjusting signals for possible differences in protein loading, detection of the housekeeping protein GAPDH was performed by stripping bound antibodies (incubation for 45 minutes at 68°C in 62.5 mmol/L Tris-HCl [pH 6.7], 100 mmol/L 2-mercaptoethanol, 2% [wt/vol] SDS), incubating membranes overnight at 4°C with anti-GAPDH immunoglobulin G (1:2000 dilution), and then incubating membranes with horseradish peroxidase–conjugated rabbit antigoat immunoglobulin G (1:5000 dilution).

2.6. Northern blotting analysis

RNA extraction, electrophoresis, and Northern blotting were conducted as described previously [16]. After staining the gels with ethidium bromide, the 28S and the 18S ribosomal RNA bands were visualized by ultraviolet transillumination and photographed to ensure that extracted RNA was intact and evenly loaded.

2.7. Statistical analysis

Data are presented as means ± SE. Variations between groups were analyzed by factorial analysis of variance followed by the Fisher protected least significant difference test. The Pearson product moment test was used to determine the correlation (r) between pairs of variables. $P < .05$ was considered to be statistically significant. StatView for Macintosh, version 5.0, from SAS Institute (Cary, NC) was used for data analysis.

3. Results

3.1. Body weight, liver relative weight, liver glycogen concentration, and plasma parameters in LETO and OLETF rats

Body weight was significantly higher in OLETF than in LETO rats at both 8 and 25 weeks of age [16]. The liver relative weight (liver weight/body weight × 100) did not change between OLETF and LETO rats aged 8 weeks (3.68% ± 0.16% vs 3.96% ± 0.11% body weight, respectively); however, OLETF rats had proportionally heavier livers than controls at 25 weeks of age (3.60% ± 0.09% vs 3.06% ± 0.05% body weight, respectively; $P < .01$). Although OLETF rats showed a tendency to have higher liver glycogen concentrations than same-aged LETO rats (20.2 ± 4.0 vs 12.9 ± 2.6 μg/mg tissue for young rats and 36.3 ± 2.3 vs 31.6 ± 1.9 μg/mg tissue for middle-aged rats), no statistically significant differences were detected. Variations in the plasma insulin, glucose, and FFA concentrations of the 4 groups of rats can be found in our previous report [16]. Briefly, plasma insulin and glucose concentrations were at normal levels at 8 weeks of age, but were significantly higher in OLETF than in LETO rats at 25 weeks of age. The plasma FFA content, however, was significantly higher in OLETF than in LETO rats at both 8 and 25 weeks of age.

3.2. Hepatic PDC and PDK activities in LETO and OLETF rats

Table 1 shows the results of enzyme activity measurements. Activated PDC activities in the liver of OLETF rats were only 29% and 54% of those in control LETO rats at 8 and 25 weeks, respectively, suggesting that pyruvate oxidative decarboxylation in OLETF rats was depressed before the onset of diabetes and remained at low levels in animals with overt insulin resistance and diabetes. Total PDC activity in the liver was similar between LETO and OLETF rats at 8 weeks of age; however, total PDC activity was significantly ($P < .001$) higher in OLETF than in LETO rats at 25 weeks. The calculated activity states (%) of the complex for the 4 groups of rats showed almost the same tendency as the activated PDC activities, being markedly

Table 1
Activated PDC and total PDC activities, complex activity state, and PDK activity in livers of young and middle-aged LETO and OLETF rats

	8 wk		25 wk	
	LETO	OLETF	LETO	OLETF
Activated PDC activity (mU/g)	89.6 ± 4.9	25.9 ± 2.4*	61.4 ± 1.5	33.0 ± 2.4*
Total PDC activity (mU/g)	746.3 ± 20.8	732.8 ± 55.1	450.7 ± 11.5	851.2 ± 48.8*
Activity state (%)	12.0 ± 0.5	3.6 ± 0.3*	13.6 ± 0.4	3.9 ± 0.2*
PDK activity (min^{-1})	0.34 ± 0.02	0.43 ± 0.03†	0.27 ± 0.02	0.40 ± 0.01*

Activities of PDC and PDK in freeze-clamped liver extracts were measured spectrophotometrically by coupling the reaction to arylamine acetyltransferase, as described in Materials and Methods. Values are means ± SE (n = 6).

* $P < .001$ vs LETO.

† $P < .01$ vs LETO.

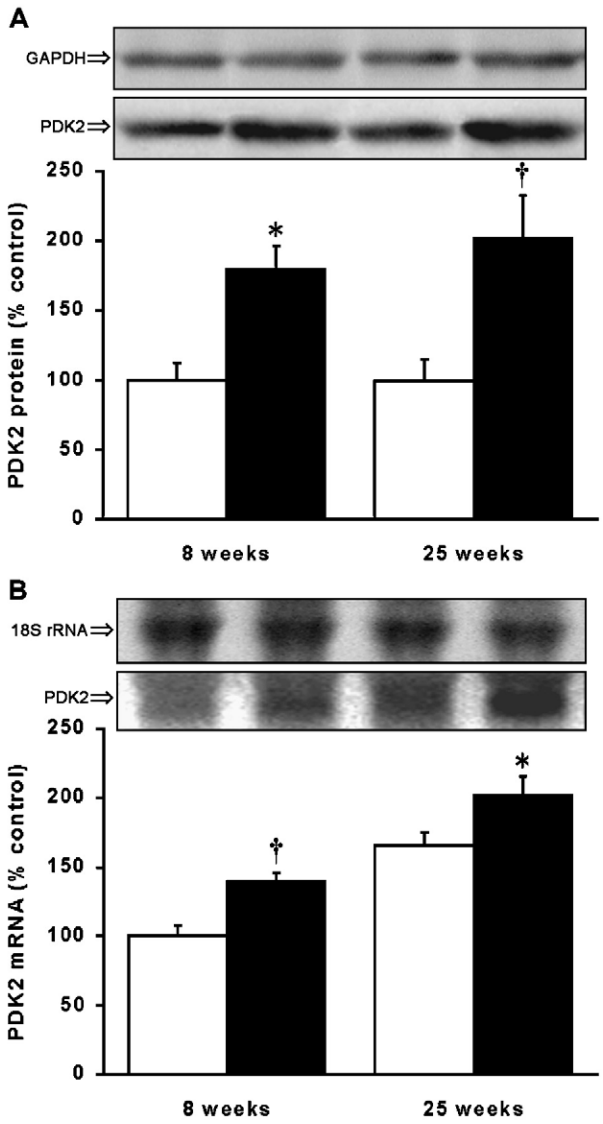

Fig. 1. Hepatic PDK2 protein and mRNA abundance in LETO and OLETF rats. Representative blots and quantitative analysis of hepatic PDK2 protein amounts (A) and mRNA expression (B) in LETO (white bars) and OLETF (black bars) rats aged 8 and 25 weeks. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting analysis, RNA extraction, agarose-formaldehyde gel electrophoresis, Northern blotting analysis, and densitometry were performed as described in Materials and Methods. Bar graphs give quantification of the relative amounts of PDK2 protein and mRNA for 6 animals in each group (means ± SE). *$P < .05$ vs LETO; †$P < .01$ vs LETO.

($P < .001$) lower in OLETF than in LETO rats at both 8 and 25 weeks of age. In addition, the activity state of PDC was inversely correlated with plasma FFA concentrations in rats aged 8 and 25 weeks ($r = -0.69$, $P < .001$). Pyruvate dehydrogenase kinase activities were significantly higher in OLETF than in LETO rats at both 8 and 25 weeks of age ($P < .01$ and $P < .001$, respectively), in agreement with the activity state of the complex.

3.3. Amounts of hepatic PDK2 and PDK4 proteins and mRNAs in LETO and OLETF rats

Hepatic amounts of PDK2 (Fig. 1A) and PDK4 (Fig. 2A) proteins were significantly greater in OLETF than in LETO rats at both 8 and 25 weeks of age. In agreement with the measured protein levels, the relative abundances of PDK2 (Fig. 1B) and PDK4 (Fig. 2B) mRNAs were significantly greater in OLETF than in control rats at both 8 and 25 weeks of age. The correlation between plasma FFA levels and the

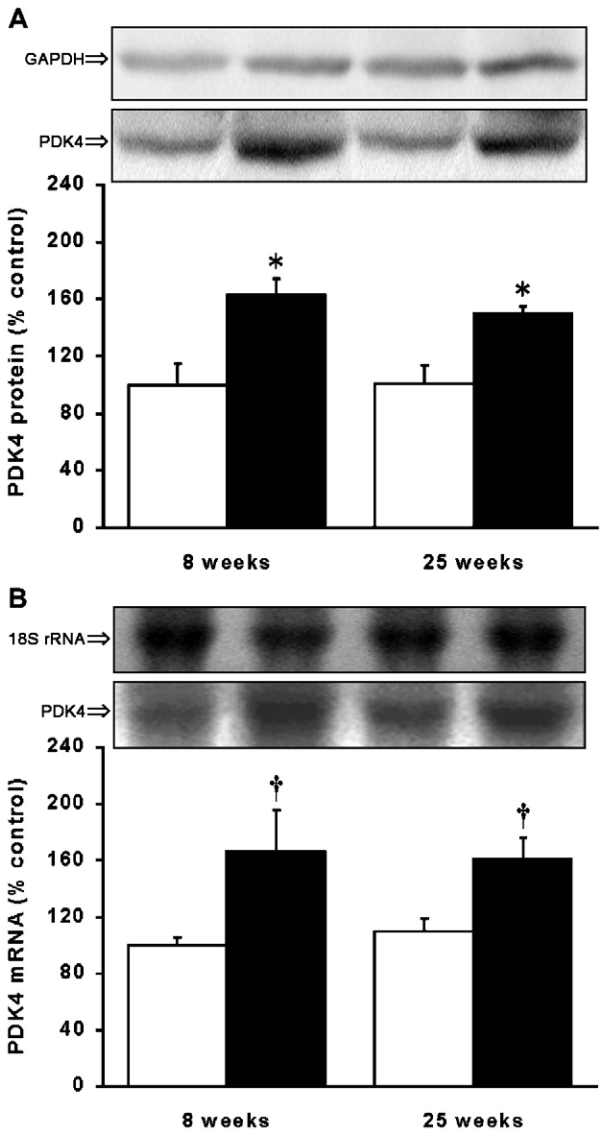

Fig. 2. Hepatic PDK4 protein and mRNA abundance in LETO and OLETF rats. Representative blots and quantitative analysis of hepatic PDK4 protein amounts (A) and mRNA expression (B) in LETO (white bars) and OLETF (black bars) rats aged 8 and 25 weeks. SDS-PAGE, Western blotting analysis, RNA extraction, agarose-formaldehyde gel electrophoresis, Northern blotting analysis, and densitometry were performed as described in Materials and Methods. Bar graphs give quantification of the relative amounts of PDK4 protein and mRNA for 6 animals in each group (means ± SE). *$P < .01$ vs LETO; †$P < .05$ vs LETO.

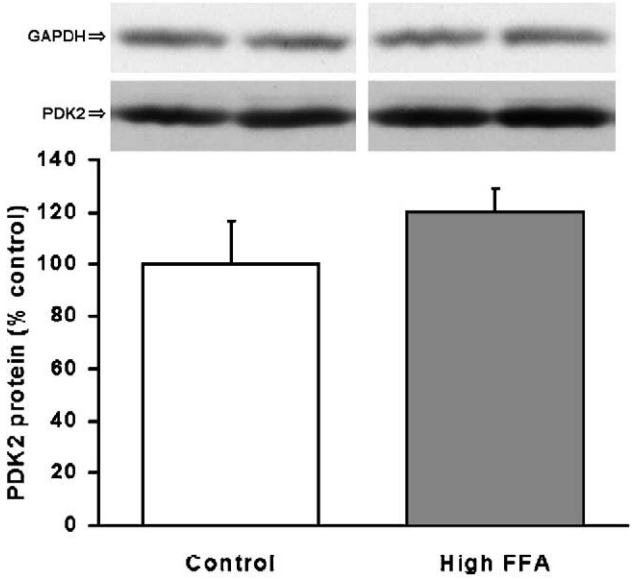

Fig. 3. Effect of high FFA levels on hepatic PDK2 protein amounts in SD rats. Representative blots and quantitative analysis of hepatic PDK2 protein amounts in control and corn oil plus heparin–injected SD rats. SDS-PAGE, Western blotting analysis, and densitometry were carried out as described in Materials and Methods. Bar graph gives a quantification of the relative amounts of PDK2 protein for 5 animals in each group (means ± SE).

abundance of mRNA encoding PDK2 in LETO and OLETF rats was $r = 0.67$ ($P < .001$), and between plasma FFA levels and the abundance of hepatic PDK4 mRNA in the same animals was $r = 0.58$ ($P < .01$).

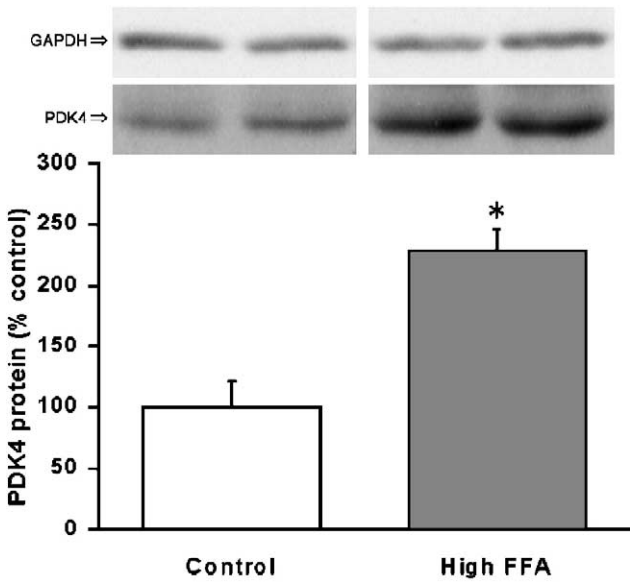

Fig. 4. Effect of high FFA levels on hepatic PDK4 protein amounts in SD rats. Representative blots and quantitative analysis of hepatic PDK4 protein amounts in control and corn oil plus heparin–injected SD rats. SDS-PAGE, Western blotting analysis, and densitometry were carried out as described in Materials and Methods. Bar graph gives a quantification of the relative amounts of PDK4 protein for 5 animals in each group (means ± SE). *$P < .01$ vs control.

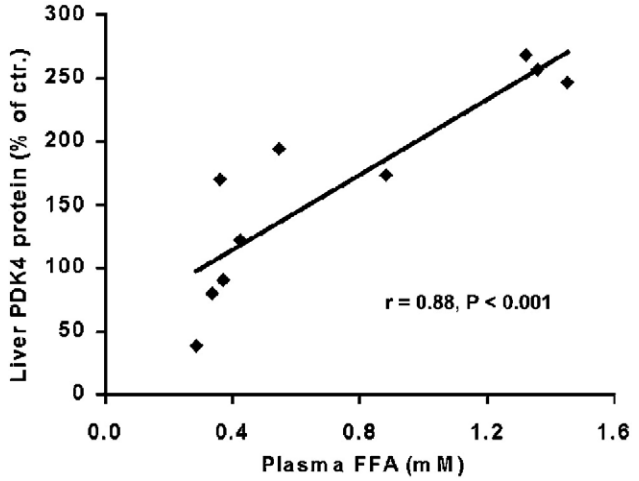

Fig. 5. Relationship between plasma FFA concentrations and hepatic PDK4 protein amounts in SD rats. Data were obtained as described in Materials and Methods. This association remains significant ($P < .001$) after omitting the apparent outliers.

3.4. Effects of artificially elevated FFA levels on the amounts of hepatic PDK2 and PDK4 proteins in SD rats

Compared with control, corn oil load followed by subcutaneous heparin injection induced significant increases in plasma FFA concentrations at the time of dissection (0.356 ± 0.022 vs 1.112 ± 0.173 mmol/L; $P < .01$). Amounts of hepatic PDK2 protein tended to be greater in rats with higher FFA levels, but with no statistical significance (Fig. 3). Amounts of PDK4 protein, instead, were markedly ($P < .01$) increased by artificial elevation of plasma FFA concentrations (Fig. 4). A significant positive correlation between plasma FFA concentrations and hepatic PDK4 protein amounts was found, as shown in Fig. 5.

4. Discussion

Mammalian PDC is a key enzyme in cellular metabolism; it irreversibly commits 3-carbon intermediates derived from the catabolism of carbohydrates or certain amino acids to acetyl-CoA conversion. In liver, the activity of PDC must be suppressed under gluconeogenic conditions (during starvation or high dietary fat consumption) when pyruvate is required to synthesize oxaloacetate. During lipogenesis, however, increased PDC activity is required to supply acetyl-CoA for fatty acid or sterol biosynthesis [26]. Reduced PDC activity in most of the body is crucial for glucose conservation when this substrate is scarce, favoring use of lipids as metabolic fuel [1] and maximizing the availability of 3-carbon compounds for gluconeogenesis [13]. However, inactivation of the complex by hyperphosphorylation is detrimental in diabetes [13].

In the present study, we examined the relationship between increased plasma FFA concentrations and PDC

activity in liver and the mechanisms by which PDC is regulated through differential expression of specific kinases in both young/normal and middle-aged/diabetic OLETF rats, as compared with LETO controls. The significant, negative correlation between hepatic PDC activity state and the plasma FFA concentration in rats aged 8 and 25 weeks suggests reduced pyruvate oxidation and augmented fat metabolism at both time points in normal and insulin-resistant animals. Up-regulation of liver PDK activity (increased PDK2 and PDK4 proteins) was found to play a decisive role in the mechanism by which PDC is phosphorylated and consequently down-regulated in OLETF rats. Furthermore, given that the relative abundance of the mRNAs encoding PDK2 and PDK4 exhibited approximately the same variation as respective protein amounts among the 4 groups of rats, regulation of the expression of these isoenzymes may occur primarily at the transcription level.

These outcomes were analogous to those of our previous study, which was focused on the regulatory mechanisms of PDC in skeletal muscle of OLETF rats [16]. Young and middle-aged animals also exhibited significantly diminished PDC activity and augmented PDK activity in skeletal muscle, indicating reduced pyruvate oxidation for the purpose of generating energy. However, unlike the case of skeletal muscle, PDK2 expression in hepatic tissue was significantly increased in young rats, suggesting the presence of a tissue-specific regulatory mechanism for this kinase.

An intriguing finding of the present study was the markedly low total PDC activity in the liver of LETO rats aged 25 weeks, as compared with the other 3 groups (Table 1). The reason for this discrepancy is not clear; however, given that the activated PDC activity of middle-aged LETO rats was lower than that in young LETO rats, we hypothesize that the amounts of hepatic PDC protein were diminished at 25 weeks of age. Further studies are required to clarify the circumstances that brought about this change.

The functional importance of the lipid-activated transcription factor PPARα for PDK4 expression in kidney and heart during starvation or WY-14,643 treatment has been documented [27,28], suggesting an important role for elevated FFA in transcriptional induction. In addition, although insulin administration may reverse the effects of chemically induced diabetes on PDK4 expression in rat skeletal muscle [11], insulin was relatively ineffective in preventing or reversing the induction of PDK4 by WY-14,643 or FFA in cultured cells [13], probably as a consequence of insulin resistance that occurs when FFA levels are elevated [29]. Therefore, in this study, it could be inferred that, despite the presence of markedly high levels of plasma insulin in OLETF rats aged 25 weeks, the impaired hormonal signaling associated with increased levels of plasma FFA was chiefly responsible for modulation of PDK4 gene expression, resulting in down-regulation of PDC activity in the liver. In OLETF rats aged 8 weeks, however, it appears that increased plasma FFA alone was responsible for the reduction in the activity state of PDC, by means of a significant increase in the PDK4 expression induced by PPARα activation.

Notwithstanding that starvation and STZ-induced diabetes have been reported to have little or no effect on PDK2 protein levels in rat skeletal muscle [10,11] or heart [22], starvation [7,8] and hyperthyroidism [7] may significantly up-regulate the expression of PDK2 in liver. Huang et al [13] also showed that WY-14,643 and the glucocorticoid dexamethasone have no effect on PDK2 message levels in cultured Morris hepatoma 7800 C1 cells, whereas a gradual inhibitory effect was evident when the same cells were treated with increasing concentrations of insulin. These reports demonstrate the physiological importance of this isoform in hepatic tissue and suggest that distinct mechanisms regulate the abundance of PDK2 and PDK4, although these isoenzymes respond similarly to insulin treatment. In the present study, we found significantly greater PDK2 expression in the liver of normal and diabetic OLETF rats, as judged against LETO controls. Therefore, both isoforms may work jointly to elevate PDK activity, with consequent down-regulation of PDC (ie, increased levels of E1α Ser264 and Ser271 phosphorylation). The presence of a statistically significant positive correlation between plasma FFA levels and the abundance of hepatic PDK2 mRNA in LETO and OLETF rats suggests that activation of PPARα might also be involved in the transcriptional regulation of this kinase.

In a parallel study, we tested whether short-term elevation in circulating FFA levels could lead to increases in hepatic PDK2 and PDK4 proteins. When compared with controls, a 3-fold artificial elevation in plasma FFA concentrations brought about a 2.3-fold increase in the amount of PDK4 protein in liver, with statistically significant direct proportionality (Fig. 5). However, the PDK2 protein content exhibited only a modest, insignificant change (1.2-fold elevation) during short-term elevation in plasma FFA, thus suggesting that the effects of PPARα activation on PDK2 and PDK4 expression favor a switch toward preferential expression of PDK4. In other words, when compared with PDK4, PDK2 expression is a slower adaptation, and thus the possibility that persistently elevated levels of circulating FFA in young and middle-aged OLETF rats signal up-regulation of hepatic PDK2 expression cannot be ruled out.

In conclusion, our findings suggest that persistently elevated levels of plasma FFA in normal and diabetic OLETF rats play an important role in stimulating PDK2 and PDK4 expression in liver. This, in turn, up-regulates PDK activity and determines increases in the phosphorylation state of the complex, thus down-regulating PDC activity. Inactivation of PDC may favor hepatic gluconeogenesis, which works unfavorably under insulin-resistant or diabetic conditions. Furthermore, reduced PDC activity in the liver and skeletal muscle [16] of young OLETF rats may be related to the development of diabetes mellitus.

Acknowledgment

This work was supported by grants-in-aid for scientific research (11670066 to Y Sato and 14370022 to Y Shimomura) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant for longevity science research (H10-025 to Y Sato) from the Ministry of Health, Labour and Welfare of Japan.

The authors gratefully acknowledge Dr Kazuya Kawano (Otsuka Pharmaceutical) who kindly provided the animals used in this study.

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