Induction of PDK4 in the heart muscle of JVS mice, an animal model of systemic carnitine deficiency, does not appear to reduce glucose utilization by the heart

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Abbreviations: BCKDC, branched-chain ketoacid dehydrogenase complex; DTT, dithiothreitol; E1, pyruvate dehydrogenase component of PDC; E2, dihydrolipoyl acetyltransferase component of PDC; E3, dihydrolipoamide dehydrogenase component of PDC; E3BP, E3-binding protein component of PDC; HA, hemagglutinin antigen; JVS, juvenile visceral steatosis; KGDC, α-ketoglutarate dehydrogenase complex; LDH, lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase

Keywords: Cardiac hypertrophy; Carnitine deficiency; Glucose uptake; Juvenile visceral steatosis (JVS) mice; Pyruvate dehydrogenase complex

Abstract

Pyruvate dehydrogenase kinase 4 (PDK4) mRNA has been reported as an up-regulated gene in the heart and skeletal muscle of carnitine-deficient juvenile visceral steatosis (JVS) mice under fed conditions. PDK4 plays an important role in the inhibition of glucose oxidation via the phosphorylation of pyruvate dehydrogenase complex (PDC). This study evaluated the meaning of increased PDK4 mRNA in glucose metabolism by investigating PDK4 protein levels, PDC activity and glucose uptake by the heart and skeletal muscle of JVS mice. PDK4 protein levels in heart and skeletal muscle of fed JVS mice were increased in accordance with mRNA levels, and protein was enriched in the mitochondria. PDK4 protein was co-fractionated with PDC in sucrose density gradient centrifugation, like PDK2 protein. However, the activities of pyruvate dehydrogenase complex (PDC) active form in the heart and skeletal muscle of fed JVS mice were similar to those in fed control mice. Fed JVS mice showed significantly higher glucose uptake in the heart and similar in the skeletal muscle compared with fed control mice. Thus, in carnitine deficiency under fed conditions, glucose was preferentially utilized in the heart as an energy source despite increased PDK4 protein levels in the mitochondria. The preferred glucose utilization may be involved in developing cardiac hypertrophy from carnitine deficiency in fatty acid oxidation abnormality.

Introduction

Juvenile visceral steatosis (JVS) mice, an animal model of systemic carnitine deficiency (SCD), showed cardiac hypertrophy and progressive cardiac dysfunction [1-6]. In SCD, heart problems including hypertrophy and arrhythmia are clinically important [7,8]. Understanding the pathophysiology of hypertrophy of JVS mice will help to prevent and treat heart problems in human SCD. Here, we tried to study the pathophysiology of hypertrophy by elucidating the role of pyruvate dehydrogenase kinase (PDK) 4, which was found as an up-regulated gene in the hypertrophied heart [9], in glucose metabolism.

JVS mice were discovered originally in the C3H.OH strain suffering from fatty liver [10]. Subsequent studies revealed that JVS mice also have hypoglycemia, hyperammonemia, growth retardation and cardiac hypertrophy, in addition to fatty liver, thus resembling the symptoms of human SCD [3,11]. JVS mice showed severely lowered carnitine levels in blood due to lowered renal reabsorption of carnitine [12,13]. Genetically, JVS mice showed a spontaneous mutation in the mouse homologue of organic cation transporter 2 (OCTN2), as well as human SCD [14,15]. Thus, JVS mice are now established as an animal model for human SCD. Cardiac hypertrophy was ameliorated by intraperitoneal administration of carnitine. Additionally, peroxisome proliferator-activated receptor alpha (PPARα) agonists [16], or lowering the fat content of the diet [17] attenuates hypertrophy, suggesting that fatty acids which cannot be metabolized are involved in its development. Antioxidants are also effective, indicating that oxidative stress plays an important role in hypertrophy [18]; however, the mechanism of hypertrophy is still not completely understood. For better understanding, differences in gene expressions between hypertrophied and normal hearts were surveyed. JVS mice showed mRNA of atrial natriuretic peptide and skeletal muscle actin up-regulated in the heart as well as the

pressure-overloaded hypertrophied heart [4]. On the other hand, JVS mice showed unchanged mRNA in β-myosin heavy chain and decreased mRNA in cardiotrophin [4,19], different from the pressure-overloaded hypertrophied heart. These results indicate that the mechanism of hypertrophy is at least partially different from that of pressure-overloaded hypertrophy. We also found that pyruvate dehydrogenase kinase (PDK) 4 mRNA, which has been reported as a down-regulated gene in the pressure-overloaded hypertrophied heart, was increased in the heart of JVS mice [9,20,21]. As noted in studies on pressure-overloaded hypertrophy, down-regulated PDK4 is involved in the increased reliance on glucose as fuel [20]. It is interesting to examine whether increased PDK4 mRNA in the JVS mice heart plays a role in glucose metabolism through pyruvate dehydrogenase complex (PDC) regulation.

PDK isoforms have been reported as four respective proteins, which are coded to four different genes. The four PDK isoforms are shown on tissue distribution, affinity to substrates, and sensitivity to inhibitors [22]. All PDK isoforms are considered to be involved in the inactivation of PDC. PDK4 has been proposed to be especially involved in PDC inactivation under fasting or diabetic conditions through transcriptional regulation [23-26]. The physical association between PDK4 and PDC has not been elucidated completely, but the remaining PDK isoforms (PDK1-3) have been shown to bind to PDC through the E2-lipoyl domain [27-29]. Recently, Wynn et al. reported that PDK4 possibly binds to PDC through E3BP-lipoyl domain [30]. It is required to show clearly the physical binding between PDK4 and PDC. In JVS mice, the activity of the PDC active form (PDCa) in the heart of carnitine-deficient mice was similar to that in control mice [9], suggesting that the increased PDK4 mRNA level is not necessarily associated with PDC inactivation.

In the present study, we examined the relationship between PDK4 protein levels and

PDC activity in the heart and skeletal muscles of JVS mice. We also observed the localization of PDK4 protein in JVS mice and the physical association between PDK4 and PDC. Glucose uptake was also measured in several JVS mouse tissues.

Methods

Animals and reagents

Control (W, *jvs*$^{+/+}$) and homozygous mutant (JVS, *jvs*$^{-/-}$) mice were obtained by cross-mating heterozygous mice or by mating homozygous mutant male and heterozygous female mice [10]. Mice residing in a closed colony originating from the C3H·OH strain were used for all experiments. Neonatal (7−28 days of age) JVS mice were treated intraperitoneally with L-carnitine·HCl (5 μmol/mouse; Sigma-Aldrich, St. Louis, MO), dissolved in physiological saline and neutralized with 0.2 M NaOH, as described previously [31]. Mice were kept in 12 h lights on-off cycle (lights-on 7:00−19:00) and had free access to laboratory chow (CE2; CLEA Japan, Inc., Tokyo, Japan) and water.

This study was approved by the Ethics Committee for Animal Experimentation at Kagoshima University, which was standardized to Japanese national guidelines for animal experiments.

Except when noted, reagents were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich (St. Louis, MO). Anti-hemagglutinin (HA) antibody and peroxidase conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Roche Diagnostics (Tokyo, Japan), respectively.

Preparation of Antibodies, and Western blotting

Anti-PDK4 antibody (αPDK4) antigen was prepared as follows. The PCR product of PDK4 from mouse heart cDNA was introduced into pET-15b expression vector (Novagen, Madison, MI) to produce His_6-tagged protein. Primers (forward primer, 5'-ATCATATGAAGCAGCTGCTGGACTTTG-3'; reverse primer, 5'-AGACCCACTTGGATCCCGTAAA-3') were utilized for PCR. These contained

mismatched sequences to produce *N*deI and *BamH*I restriction enzyme sites (underlined above). Plasmids containing the PCR product were transformed in BL21 (DE3) pLysS cells. The purified protein was mixed with TiterMax Gold (CytRx, Norcross, GA) in accordance with the manufacturer's protocol, and injected into a rabbit. This gave serum reacting specifically against recombinant mouse PDK4 proteins (Fig. 2 A).

Anti-PDK2 antibody (Drs. P. Wu and R.A. Harris, Indiana University, IN) [24], and anti-branched chain α-ketoacid dehydrogenase complex (BCKDC) antibody (Dr. Y. Shimomura, Nagoya Institute of Technology, Japan) [32] were kindly donated. Anti-PDC and anti-α−ketoglutarate dehydrogenase complex (KGDC) antibodies were prepared [33].

Mouse PDK1, PDK2 and PDK4 proteins containing hemagglutinin (HA) tag at the carboxyl-terminal region were produced. The cDNA clones for mouse PDK1 (register number, IMAGp998K234710Q2) and PDK2 (register number, IMAGp998N044710Q2) were obtained from RZPD Deutsches Ressourcenzentrum (Berlin, Germany). The cDNA clone for PDK4 was prepared from a mouse heart cDNA [9]. PCR products (PDK1, 5'-GACCATGAGGCTGGCAAGGCTG-3' and 5'-GCTCGAGTTAAGCGTAGTCTG-GGACGTCGTATGGGTAAGAGCTTCGGAATGTGGT-3'; PDK2, 5'-GACCATGCGT-TGGGTCCGGGC-3' and 5'-GCTCGAGCTAAGCGTAGTCTGGGACGTCGTATGGGT-AGCTGACCCGA-3'; PDK4, 5'-GACCATGAAGGCAGCCCGCTTC-3' and 5'-GCTCGAGTCAAGCGTAGTCTGGGACGTCGTATGGGTACACTGCCAGCTTCTC CTTC-3') were subcloned into a eukaryotic expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA). The underlined portions of the primers above each correspond to a region of HA tag. The plasmids were transfected into COS cells by the calcium-phosphate method [34]. The recombinant proteins were homogenized by buffer A (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

For Western blot analysis, samples were treated with an equal volume of SDS buffer [0.12 M Tris-HCl pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, and 0.4% (w/v) bromphenol blue, 200 mM DTT]. Electrophoresis (10% SDS-PAGE) and transfer followed the manufacturer's instructions (Bio-Rad, Hercules, CA). Nitrocellulose membrane was purchased from Bio-Rad. An ECL kit (PIERCE, Rockford, IL) was used to detect immunoreactive materials. The detected signals were quantified by ChemiDoc XRS and Quantity One software (Bio-Rad).

Real-time PCR for quantification of PDK2, PDK4 and PDP1mRNAs

Total RNA was isolated from anesthetized mice (pentobarbital: 100 mg/kg body weight) using the method of Chomczynski and Sacchi [35]. Isolated RNA was treated with DNAase (Ambion Inc., Austin, TX) to delete genomic contamination. First-strand cDNA was synthesized using 5 μg total RNA and oligo-$(dT)_{12-18}$ primer following the manufacturer's instructions (Invitrogen). Real-time quantitative PCR was performed using SYBR-Green on a Thermal Cycler Dice Real Time System (Takara Bio Inc., Otsu, Japan). The cyclophylin gene was used as an internal control for all samples. The cycle threshold number (Ct) at which amplification entered the exponential phase was determined for each gene under investigation. Gene expression levels were analyzed using the delta-delta Ct method, determining the target gene expression relative to an internal control and relative to control individual samples. The primers used were as follows: 5’-CAGCGTGTCTGATGTGGTGA-3’ and 5’-CAGGTCAGGGGAAGCCATG-3’ for PDK2 [36]; 5’-TTTGGTGGAGTTCCATGAGAA-3’ and 5’-GAACTTTGACCAGCGTGTCT-3’ for PDK4 [9]; 5’-ACTGGTATGCATCACCAACAG-3’ and 5’-ACACTGATGACATCTTTGCTCT-3’ for

pyruvate dehydrogenase phosphatase catalytic subunit 1 (PDP1) (NM_001033453) [37]; 5'-GGTGGAGAGCACCAAGACAGA-3' and 5'-GCCGGAGTCGACAATGATG-3' for cyclophilin [38]

Measurement of PDC activity of active and total forms [39,40]

For the active form, hearts and skeletal muscles were taken from anesthetized mice and immediately freeze-clamped with Wolleneberger tongs pre-cooled in liquid nitrogen. The frozen tissues were homogenized with buffer B (0.25 M sucrose, 5 mM Tris-HCl and 2 mM EGTA, pH 7.4, containing 50 mM sodium fluoride and 10 mM sodium dichloroacetate).

For the total form, fresh tissues were disrupted with a polytron homogenizer in buffer B. Mitochondria were prepared by differential centrifugation and then incubated (30 min at 30^{o}C) in buffer C (120 mM KCl, 20 mM Tris-HCl, 5 mM potassium phosphate, 2 mM EDTA, pH 7.4) containing 10 mM carbonyl cyanide m-chlorophenylhydrazone, a respiratory inhibitor. The incubated mitochondria were precipitated by centrifugation, and frozen at -80^{o}C. The mitochondria were then thawed (30^{o}C) with buffer D (50 mM potassium phosphate, 2 mM EGTA, pH 7.0) containing 1.0% (v/v) Triton X-100. Both forms of PDC activities were measured by determining $^{14}CO_2$ production from [1-^{14}C]-pyruvate [39]. Citrate synthase activity [41] was determined spectrophotometrically as an indication of mitochondrial recovery. The unit of PDC activity is defined as 1 μmol of substrate converted into product per min at 37^{o}C. Total PDC activity was calculated as: [PDC activity in mitochondria fraction/citrate synthase activity (mitochondria fraction)] x [citrate synthase activity (homogenate fraction)/mg protein of tissue] [40].

Tissue homogenization and mitochondria isolation

Tissues from anesthetized mice were taken and frozen until use. The tissues were homogenized by buffer E [30 mM HEPES, 1 mM EDTA, 0.15 M KCl, 3 mM DTT, 0.1% (v/v) Triton X-100, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 0.1 mg/ml pefablock, pH7.4] with a Potter-Elvehjem homogenizer. For mitochondria isolation, fresh hearts from anesthetized mice were homogenized with buffer F (0.25 M sucrose and 10 mM Tris-HCl, pH7.4). The homogenate was filtrated (75 μm mesh) and centrifuged at 600 x g for 10 min at 4^{o}C. The supernatant was centrifuged again at 8,000 x g for 10 min at 4^{o}C. The precipitate suspended in buffer E was fractionated to obtain the mitochondria fraction with a sucrose gradient (1.10−1.56 M sucrose in 10 mM Tris-HCl, pH 7.4) at 82,000 x g for 200 min at 4^{o}C. Mitochondria fractionation was evaluated by measuring the activities of lactate dehydrogenase (commercial kit; Wako Pure Chemical Industries, Japan) and citrate synthase [41].

Fractionation of α-ketoacid dehydrogenase complexes with sucrose gradient techniques

The frozen hearts were homogenized with buffer A. The homogenate (Ho) was centrifuged at 600 x g for 10 min at 4^{o}C, and the supernatant (S1) was centrifuged at 20,000 x g for 10 min at 4^{o}C to obtain the supernatant (S2) and the precipitate (P2). The precipitate (P2) was suspended in buffer G [30 mM HEPES, 1 mM EDTA, 0.15 M KCl, 3 mM DTT, 0.1% (v/v) Triton X-100]. The suspension was ultra-centrifuged at 100,000 x g for 90 min at 4^{o}C, and divided into supernatant (S3) and precipitate (P3). This precipitate (P3) was suspended in buffer G and ultra-centrifuged at 100,000 x g for 60 min. This precipitate was designated P4.

The P4 fraction was suspended in buffer G without Triton X-100, and was applied

to the top of a sucrose gradient cushion. The sucrose gradient was composed of 4 ml of 1.46 M sucrose and 34 ml of 0.29−0.88 M sucrose in buffer G without Triton X-100. After ultra-centrifugation (50,000 x g for 15 h), 10 drops (about 1.1 ml) were collected from the bottom for fractionation.

2-Deoxy-glucose uptake [42-44]

2-Deoxy-glucose uptake was analyzed in tissues using intraperitoneal injections of 2 μCi ([1-^{3}H]2-deoxy-D-glucose)/20 g body weight. The isotope, [1-^{3}H]2-deoxy-glucose ([^{3}H]2-DG; 3 Ci/mmol, 1.0 mCi/ml; Moravek Biochemicals, CA), was diluted with 50 times volume (100 μl) of 0.9% NaCl. Mice were killed by decapitation 30 min after injection. The heart, femoral skeletal muscle, epididymal fat, liver and brain were immediately excised, weighed and homogenized with 6% (w/v) $HClO_4$ in 80 mM triethanolamine. The homogenates were centrifuged at 12,000 xg for 5 min. ^{3}H radioactivity levels of the supernatant from these tissues were measured using a liquid scintillator (Aquasol 2; Perkin Elmer Japan, Japan). Plasma glucose was measured with a commercial kit (Glucose CII-test; Wako, Japan).

Statistical analysis

Values are shown as the means ± standard deviation (SD). Data were analyzed by unpaired Student's *t* test. $P < 0.05$ was considered significant.

Results

PDK4 mRNA and protein levels in fed and 24h-fasting JVS mice

To determine PDK4 mRNA and protein levels in fed and fasting JVS adult mice (2−3 months of age), we performed real-time PCR and Western blot (Figs. 1 and 2). Under fed conditions, JVS mice showed significantly higher levels of PDK4 mRNA in the heart compared with controls, but no significant differences in PDK2 mRNA levels between control and JVS mice (Fig. 1). Control mice showed significantly higher levels of PDK4 mRNA in the heart under fasting than fed conditions. JVS mice showed no significant differences in PDK4 mRNA levels of hearts between fed and fasting conditions. Messenger RNA of PDP1, another regulator of PDC, was significantly lower in fed JVS mice than in fed control mice (Fig. 1). PDK4 and PDK2 protein levels were detected by the respective antibodies (Fig. 2). JVS mice under fed conditions showed significantly higher levels of PDK4 protein in the heart, skeletal muscle, liver and kidney than control mice (Fig. 2B). No PDK4 proteins were detected in the brain of either JVS or control mice in this analysis. On the other hand, in fed control mice, PDK2 protein was detected, mainly in the heart, but also faintly in the skeletal muscle, liver, kidney and brain. As shown in Fig. 2C, we compared PDK4 and PDK2 protein levels under fed and fasting conditions. PDK4 protein levels in the hearts of fed JVS mice were significantly higher than in fed control mice, and not significantly different from 24h-fasting control mice (Fig. 2C). After 24 h fasting, both JVS and control mice showed a significant increase in PDK4 protein in the heart compared with the respective fed mice. In skeletal muscle, fed JVS mice showed remarkably higher PDK4 protein levels than fed control mice, which had undetectable levels (Fig. 2C). Skeletal muscle PDK4 protein levels in fed JVS mice were not significantly different from those of 24h-fasting control mice.

PDC activity of fed and 24h-fasting JVS mice

We measured the activities of PDC active form (PDCa) and total PDC (PDCt) in the heart and skeletal muscle. As shown in Table 1, PDCa activities in the heart and skeletal muscle of JVS mice were not significantly different from those of fed control mice. Fed JVS mice showed significantly lower PDCt activity in heart than fed control mice. After 24 h fasting, PDCa activity significantly decreased in the heart and skeletal muscle of JVS and control mice. Heart PDCt activity in 24h-fasting control mice was significantly lower than in fed control mice.

Cellular distribution and physical association with PDC of PDK4 protein

To know why increased PDK4 protein in JVS mice does not contribute to PDC inactivity in the heart and skeletal muscle, we performed experiments and investigated: 1) whether increased PDK4 protein is enriched in the mitochondria of JVS mice, and 2) whether PDK4 protein is physically associated with PDC.

To resolve the first question, we purified mitochondria from the hearts of fed JVS and control mice by using ultra-centrifugation and a sucrose gradient (Fig. 3A). The mitochondria fraction showed low contamination of cytosolic components because of very little LDH activity, a cytosolic protein (Fig. 3A). PDK4 protein was enriched in the mitochondria fraction of control and JVS mice, indicating that increased PDK4 protein was transported in mitochondria. For the second question, PDK4 protein from the heart of fed JVS mice was precipitated after 100,000 x g centrifugation (as with PDK2 and PDC proteins) (Fig. 3B). To examine whether PDK4 protein is bound to PDC, we further fractionated the 100,000 x g precipitate through sucrose density gradient centrifugation. PDK4 protein showed a similar profile to that of PDK2 in the sucrose density gradient (Fig.

3C). Apparently, PDK2 and PDK4 appeared to be eluted in fractions showing PDC, but not in fractions showing KGDC and BCKDC (Fig. 3C).

2-DG uptake

There were no significant differences in plasma glucose between fed control and JVS mice (161 ± 16 versus 165 ± 9 mg/dl, n = 6, respectively). 2-DG uptake was measured in control and JVS mice under fed conditions (Fig. 4). JVS mice showed significantly larger 2-DG uptake in the heart and liver than control mice. There were no significant differences in 2-DG uptake in the brain, white adipose tissue and skeletal muscle between control and JVS mice.

Discussion

The present study revealed that PDK4 protein, as well as its mRNA level, was increased in the heart and skeletal muscle of carnitine-deficient mice under fed conditions compared with control mice (Figs. 1 and 2). Moreover, PDP1mRNA, a regulator involved in activation of PDC through its dephsphorylation, was significantly lower in fed JVS mice than in fed control mice (Fig. 1)[37]. On the other hand, there were no significant differences in PDCa activity in the heart and skeletal muscle between control and JVS mice (Table 1). These results suggest that PDK4 is not directly involved in PDC regulation under fed conditions in carnitine deficiency. And also, PDP1 may not play a role in maintaining activity of PDC in fed JVS mice. In the literature, PDK4 protein itself has been considered to play an important role in inactivating PDC under physiological and pathological conditions [20,21,24-26]. Using PDK4 knock-out mice [45], PDK4 in tissues other than the liver has been shown to be important during fasting for regulation of PDC activity and glucose homeostasis [45]. However, our study has shown that there may be an underlying mechanism that increases PDK4 protein in the heart and skeletal muscle of JVS mice to be less effective in suppressing PDC activity. As a possible mechanism, increased PDK4 protein may not fully interact with PDC in the mitochondria, resulting from an increase of the free form of PDK4. As shown in Fig. 3C, PDK4, as well as PDK2, appeared to be eluted in the fraction showing PDC, indicating that PDK4 binds physically to PDC. In the JVS heart, increased PDK4 was enriched in mitochondria, where it might interact with PDC (Fig. 3). For other PDK isoforms, the binding of PDK1-3 to PDC is influenced by the ratios of NADH to NAD and/or acetyl-CoA to CoA [27-29]. As a possible explanation, PDK4-PDC complex formation may be modulated intra-mitochondrially like other PDK-PDC complexes, depending on the values of [NADH/NAD] and/or [acetyl

CoA/CoA] [27-29]. In carnitine-deficient tissues, disturbance of fatty acid oxidation may result in low NADH/NAD and/or low acetyl CoA/CoA, which can affect the binding of PDK4-PDC. PDK4 has been thought to be regulated at the transcriptional step by fatty acids and/or hormones, including insulin and glucocorticoids [26,46,47]. Additionally, the present study supports that PDK4 is involved into PDC regulation at the post-transcriptional step as well as other PDKs [22,30]. Although it did not reveal the mechanism, the sucrose density gradient experiment demonstrated that PDK4 is tightly bound to the pyruvate dehydrogenase complex from the hearts of JVS mice. This is important because there is literature [28] indicating that PDK4 does not bind to the pyruvate dehydrogenase complex as tightly as the other PDKs.

We have reported that the respiratory quotient in fed JVS mice is greater than 0.9, which is similar to fed control mice, suggesting that carbohydrate is the main energy source [48]. In the present study, glucose transport was examined in control and JVS mice (Fig. 4). JVS mice showed significantly larger 2-DG uptake, corresponding to glucose uptake, in the liver and heart than the control. This finding is consistent with the other results obtained in this study showing that PDC is active in the heart and skeletal muscles of fed JVS mice (Table 1). This increased glucose uptake may compensate for the lower utilization of fatty acids in carnitine-deficient JVS mice. This assumption is consistent with previous reports on energy states in the heart and skeletal muscles in JVS mice [9,49]. These reports have noted that ATP levels are maintained even in the carnitine-deficient heart and skeletal muscles [9,49]. In the heart suffering from fatty acid oxidation abnormality due to carnitine deficiency, the preferential shift in energy from fatty acid to carbohydrate is required to suppress atrophy of cardiac cell. Moreover, the increased uptake of glucose which is induced by insulin-dependent and/or -independent manner may

be involved in development of the cardiac hypertrophy [50]. To reveal the role of the increased glucose uptake in development of the cardiac hypertrophy, further studies are required. In the pressure-overloaded heart, another type of hypertrophy, decreased PDK4 protein is associated with lowered suppression of PDC and enhanced glucose uptake, indicating that glucose oxidation is enhanced in place of fatty acid oxidation [20,21]. In hypertrophy due to carnitine deficiency, 2DG uptake, corresponding to glucose uptake, in the heart is also increased. This suggests that glucose utilization as a preferred energy source is a common mechanism developing cardiac hypertrophy of carnitine deficiency or pressure overload.

In conclusion, our data demonstrate that increased PDK4 protein in the adult phase of fed carnitine-deficient mice does not significantly affect PDC inactivation, despite increased PDK4 protein in mitochondria. We have shown that PDC is a physiological substrate for PDK4 protein, based on co-fractionation of PDK4 protein with PDC as well as PDK2 protein. Further experiments are needed to clarify how PDC is protected from its inactivation and glucose uptake is enhanced, especially in the hypertrophied heart under carnitine deficiency.

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References

[1] M. Horiuchi, H. Yoshida, K. Kobayashi, K. Kuriwaki, K. Yoshimine, M. Tomomura, T. Koizumi, H. Nikaido, J. Hayakawa, M. Kuwajima, T. Saheki, Cardiac hypertrophy in juvenile visceral steatosis (jvs) mice with systemic carnitine deficiency, FEBS. Lett. 326 (1993) 267-271.

[2] K. Yoshimine, M. Horiuchi, S. Suzuki, K. Kobayashi, J.M. Abdul, M. Masuda, M. Tomomura, Y. Ogawa, H. Itoh, K. Nakao, M. Osame, T. Saheki, Altered expression of atrial natriuretic peptide and contractile protein genes in hypertrophied ventricle of JVS mice with systemic carnitine deficiency, J. Mol. Cell. Cardiol. 29 (1997) 571-578.

[3] M. Kuwajima, K. Lu, M. Sei, A. Ono, M. Hayashi, K. Ishiguro, K. Ozaki, K. Hotta, K. Okita, T. Murakami, J. Miyagawa, I. Narama, H. Nikaido, J. Hayakawa, H. Nakajima, M. Namba, T. Hanafusa, Y. Matsuzawa, K. Shima, Characteristics of cardiac hypertrophy in the juvenile visceral steatosis mouse with systemic carnitine deficiency, J. Mol. Cell. Cardiol. 30 (1998) 773-781.

[4] M.A. Jalil, M. Horiuchi, M. Nomoto, K. Kobayashi, T. Saheki, Catecholamine metabolism inhibitors and receptor blockades only partial suppress cardiac hypertrophy of Juvenile Visceral Steatosis mice with systemic carnitine deficiency, Life Sci. 64 (1999) 1137-1144.

[5] M. Kuwajima, M. Horiuchi, H. Harashima, K. Lu, M. Hayashi, M. Sei, K. Ozaki, T. Kudo, H. Kamido, A. Ono, T. Saheki, K. Shima, Cardiomegaly in the juvenile visceral steatosis (JVS) mouse is reduced with acute elevation of heart short-chain acyl-carnitine level after L-carnitine injection, FEBS Lett. 443 (1999) 261-266.

[6] R. Takahashi, T. Asai, H. Murakami, R. Murakami, M. Tsuzuki, Y. Numaguchi, H. Matsui, T. Murohara, K. Okumura, Pressure overload-induced cardiomyopathy in

heterozygous carrier mice of carnitine transporter gene mutation, Hypertension 50 (2007) 497-502.

[7] N. Longo, C. Amat Di San Filippo, M. Pasquali, Disorders of carnitine transport and the carnitine cycle, Am. J. Med. Genet. 142C (2006) 77-85.

[8] E. Gilbert-Barness, L.A. Barness, Pathogenesis of cardiac conduction disorders in children genetic and histopathologic aspects, Am. J. Med. Genet. 140A (2006) 1993-2006.

[9] M. Horiuchi, K. Kobayashi, M. Masuda, H. Terazono, T. Saheki, Pyruvate dehydrogenase kinase 4 mRNA is increased in the hypertrophied ventricles of carnitine-deficient juvenile visceral steatosis (JVS) mice, Biofactors 10 (1999) 301-309.

[10] T. Koizumi, H. Nikaido, J. Hayakawa, A. Nonomura, T. Yoneda, Infantile disease with microvesicular fatty infiltration of viscera spontaneously occurring in the C3H-H-2^{o} strain of mouse with similarities to Reye's syndrome, Lab. Anim. 22 (1988) 83-87.

[11] M. Kuwajima, N. Kono, M. Horiuchi, Y. Imamura, A. Ono, Y. Inui, S. Kawata, T. Koizumi, J. Hayakawa, T. Saheki, S. Tarui, Animal model of systemic carnitine deficiency: analysis in C3H-H-2^{o} strain of mouse associated with juvenile visceral steatosis, Biochem. Biophys. Res. Commun. 174 (1991) 1090-1094.

[12] M. Horiuchi, K. Kobayashi, S. Yamaguchi, N. Shimizu, T. Koizumi, H. Nikaido, J. Hayakawa, M. Kuwajima, T. Saheki, Primary defect of juvenile visceral steatosis (jvs) mouse with systemic carnitine deficiency is probably in renal carnitine transport system, Biochim. Biophys. Acta 1226 (1994) 25-30.

[13] M. Horiuchi, K. Kobayashi, N. Asaka, T. Saheki, Secondary abnormality of carnitine biosynthesis results from carnitine reabsorptional system defect in juvenile visceral steatosis mice, Biochim. Biophys. Acta 1362 (1997) 263-268.

[14] K. Lu, H. Nishimori, Y. Nakamura, K. Shima, M. Kuwajima, A missense mutation of

mouse OCTN2, a sodium-dependent carnitine cotransporter, in the juvenile visceral steatosis mouse, Biochem. Biophys. Res. Commun. 252 (1998) 590-594.

[15] J. Nezu, I. Tamai, A. Oku, R. Ohashi, H. Yabuchi, N. Hashimoto, H. Nikaido, Y. Sai, A. Koizumi, Y. Shoji, G. Takada, T. Matsuishi, M. Yoshino, H. Kato, T. Ohura, G. Tsujimoto, J. Hayakawa, M. Shimane, A. Tsuji, Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter, Nat. Genet. 21 (1999) 91-94.

[16] T. Asai, K. Okumura, R. Takahashi, H, Matsui, Y. Numaguchi, H. Murakami, R. Murakami, T. Murohara, Combined therapy with PPARα agonist and L-carnitine rescues lipotoxic cardiomyopathy due to systemic carnitine deficiency, Cardiovasc. Res. 70 (2006) 566-577.

[17] M.A. Jalil, M. Horiuchi, M. Wakamatsu, M.X. Li, L. Begum, K. Suzuki, K. Kobayashi, T. Oka, T. Saheki, Attenuation of cardiac hypertrophy in carnitine-deficient juvenile visceral steatosis (JVS) mice achieved by lowering dietary lipid, J. Biochem. 139 (2006) 263-270.

[18] R. Takahashi, K. Okumura, H. Matsui, Y. Saburi, H. Kamiya, K. Matsubara, T. Asai, M. Ito, T. Murohara, Impact of α-tocopherol on cardiac hypertrophy due to energy metabolism disorder: the involvement of 1,2-diacylglycerol, Cardiovasc. Res. 58 (2003) 565-574.

[19] G. Yoshida, M. Horiuchi, K. Kobayashi, M.D. Jalil, M. Iijima, S. Hagihara, N. Nagao, T. Saheki, The signaling pathway of cardiotrophin-1 is not activated in hypertrophied ventricles of carnitine-deficient juvenile visceral steatosis (JVS) mice, In Vivo 14 (2000) 401-405.

[20] M.E. Young, F.A. Laws, G.W. Goodwin, H. Taegtmeyer, Reactivation of peroxisome

proliferator-activated receptor α is associated with contractile dysfunction in hypertrophied rat heart, J. Biol. Chem. 276 (2001) 44390-44395.

[21] A. Planavila, J.C. Laguna, M. Vázquez-Carrera, Nuclear factor-κB activation leads to down-regulation of fatty acid oxidation during cardiac hypertrophy, J. Biol. Chem. 280 (2005) 17464-17471.

[22] M.M. Bowker-Kinley, W.I. Davis, P. Wu, R.A. Harris, K.M. Popov, Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex, Biochem. J. 329 (1998) 191-196.

[23] J. Rowles, S.W. Scherer, T. Xi, M. Majer, D.C. Nickle, J.M. Rommens, K.M. Popov, R.A. Harris, N.L. Riebow, J. Xia, L. Tsui, C. Bogardus, M. Prochazka, Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human, J. Biol. Chem. 271 (1996) 22376-22382.

[24] P. Wu, J. Sato, Y. Zhao, J. Jaskiewicz, K.M. Popov, R.A. Harris, Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart, Biochem. J. 329 (1998) 197-201.

[25] Y.I. Kim, F.N. Lee, W.S. Choi, S. Lee, J.H. Youn, Insulin regulation of skeletal muscle PDK4 mRNA expression is impaired in acute insulin-resistant states, Diabetes 55 (2006) 2311-2317.

[26] P. Wu, J.M. Peters, R.A. Harris, Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor α, Biochem. Biophys. Res. Commun. 287 (2001) 391-396.

[27] K.M. Popov, Regulation of mammalian pyruvate dehydrogenase kinase, FEBS Lett. 419 (1997) 197-200.

[28] A. Tuganova, I. Boulatnikov, K.M. Popov, Interaction between the individual

isoenzymes of pyruvate dehydrogenase kinase and the inner lipoyl-bearing domain of transacetylase component of pyruvate dehydrogenase complex, Biochem. J. 366 (2002) 129-136.

[29] M. Kato, J.L. Chuang, S. Tso, R.M. Wynn, D.T. Chuang, Crystal structure of pyruvate dehydrogenase kinase 3 bound to lipoyl domain 2 of human pyruvate dehydrogenase complex, EMBO J. 24 (2005) 1763-1774.

[30] R.M. Wynn, M. Kato, J.L. Chuang, S. Tso, J. Li, D.T. Chuang, Pyruvate dehydrogenase kinase-4 structures reveal a metastable open conformation fostering robust core-free basal activity, J. Biol. Chem. 283 (2008) 25305-25315.

[31] M. Horiuchi, K. Kobayashi, M. Tomomura, M. Kuwajima, Y. Imamura, T. Koizumi, H. Nikaido, J. Hayakawa, T. Saheki, Carnitine administration to juvenile visceral steatosis mice corrects the suppressed expression of urea cycle enzymes by normalizing their transcription, J. Biol. Chem. 267 (1992) 5032-5035.

[32] K.M. Popov, Y. Shimomura, R.A. Harris, Purification and comparative study of the kinases specific for branched chain α-ketoacid dehydrogenase and pyruvate dehydrogenase, Protein Expr. Purif. 2 (1991) 278-286.

[33] S. Matuda, T. Shirahama, T. Saheki, S. Miura, M. Mori, Purification and immunochemical studies of pyruvate dehydrogenase complex from rat heart, and cell-free synthesis of lipoamide dehydrogenase, a component of the complex, Biochim. Biophys. Acta 741 (1983) 86-93.

[34] J. Kirsch, J. Kuhse, H. Betz, Targeting of glycine receptor subunits to gephyrin-rich domains in transfected human embryonic kidney cells, Mol. Cell. Neurosci. 6 (1995) 450-461.

[35] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium

thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156-159.

[36] K.M. Popov, N.Y. Kedishvili, Y. Zhao, Y. Shimomura, D.W. Crabb, R.A. Harris, Primary structure of pyruvate dehydrogenase kinase establishes a new family of eukaryotic protein kinases, J. Biol. Chem. 268 (1993) 26602-26606.

[37] B. Huang, P. Wu, K.M. Popov, R.A. Harris, Starvation and diabetes reduce the amount of pyruvate dehydrogenase phosphatase in rat heart and kidney, Diabetes 52 (2003) 1371-1376.

[38] Y. Minokoshi, T. Alquier, N. Furukawa, Y. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferre, M.J. Birnbaum, B.J. Stuck, B.B. Kahn, AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus, Nature 428 (2004) 569-573.

[39] S.C. Dennis, M. DeBuysere, R. Scholz, M.S. Olson, Studies on the relationship between ketogenesis and pyruvate oxidation in isolated rat liver mitochondria, J. Biol. Chem. 253 (1978) 2229-2237.

[40] G.S. Denyer, D. Lam, G.J. Cooney, I.D. Caterson, Effect of starvation and insulin in vivo on the activity of the pyruvate dehydrogenase complex in rat skeletal muscles, FEBS Lett. 250 (1989) 464-468.

[41] H.G. Coore, R.M. Denton, B.R. Martin, P.J. Randle, Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones, Biochem. J. 125 (1971) 115-127.

[42] G.J. Cooney, I.D. Caterson, E.A. Newsholme, The effect of insulin and noradrenaline on the uptake of 2-[1-^{14}C]deoxyglucose in vivo by brown adipose tissue and other glucose-utilising tissues of the mouse, FEBS Lett. 188 (1985) 257-261.

[43] Y. Ohira, S. Ishine, I. Tabata, H. Kurata, T. Wakatsuki, S. Sugawara, W. Yasui, H. Tanaka, Y. Kuroda, Non-insulin and non-exercise related increase of glucose utilization in

rats and mice, Jap. J. Physiol. 44 (1994) 391-402.

[44] R.M. Moadel, A.V. Nguyen, E.Y. Lin, P. Lu, J. Mani, M.D. Blaufox, J.W. Pollard, E. Dadachova, Positron emission tomography agent 2-deoxy-2-[^{18}F]fluoro-D-glucose has a therapeutic potential in breast cancer, Breast Cancer Res. 5 (2003) R199-R205.

[45] N.H. Jeoung, P. Wu, M.A. Joshi, J. Jaskiewicz, C.B. Bock, A.A. DePaoli-Roach, R.A. Harris, Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homeostasis during starvation, Biochem. J. 397 (2006) 417-425.

[46] Z. Nahle, M. Hsieh, T. Pietka, C.T. Coburn, P.A. Grimaldi, M.Q. Zhang, D. Das, N. Abumrad, CD36-dependent regulation of muscle FoxO1 and PDK4 in the PPARd/b-mediated adaptation to metabolic stress, J. Biol. Chem. 283 (2008) 14317-14326.

[47] S. Connaughton, F. Chowdhury, R.R. Attia, S. Song, Y. Zhang, M.B. Elam, G.A. Cook, E.A. Park, Regulation of pyruvate dehydrogenase kinase isoform 4 (PDK4) gene expression by glucocorticoids and insulin, Mol. Cell. Endocrinol. (in press)

[48] M.X. Li, G. Yoshida, M. Horiuchi, K. Kobayashi, T. Saheki, Prolonged effect of single carnitine administration on fasted carnitine-deficient JVS mice regarding their locomotor activity and energy expenditure, Biochim. Biophys. Acta 1761 (2006) 1191-1199.

[49] A.C. Knapp, L. Todesco, M. Torok, K. Beier, S. Kraehenbuehl, Effect of carnitine deprivation on carnitine homeostasis and energy metabolism in mice with systemic carnitine deficiency, Ann. Nutr. Metab. 52 (2008) 136-144.

[50] L. Bertrand, S. Horman, C. Beauloye, J.-L. Vanoverschelde, Insulin signalling in the heart, Cardiovasc. Res. 79 (2008) 238-248.

Fig. 1. Quantitative real-time PCR analysis of PDK2, PDK4 and PDP1 in hearts of JVS (gray column) and control (open column) mice under fed and fasting conditions. Gene expression ratios were determined by real-time PCR. The relative values to cyclophilin of fed control mice are set at 1.0. The number of mice used was 4 for each experiment. Values are shown as the means + SD. * denotes $P < 0.05$ versus control mice under the respective feeding conditions. † denotes $P < 0.05$ versus the respective fed mice.

Fig. 2. Western blot analysis. (A) Specificity of the antibodies (αPDK2 and αPDK4) was shown using homogenates (10 μg protein) from COS cells expressing recombinant HA-tagged PDK1, 2 and 4 proteins. "Mock" denotes homogenate from cells transfected with an empty insert vector (pcDNA 3.1). Anti-HA antibody (αHA) detects recombinant proteins and non-specific signals (#). (B) Levels of PDK2 and PDK4 proteins in various organs, including the heart (H), skeletal muscle (SM), liver (L), kidney (K) and brain (Br), of control (W) and JVS (J) mice under fed conditions. Ten μg protein was loaded in each lane. (C) Levels of PDK2 and PDK4 proteins in the heart and skeletal muscle (SM) of control (W) and JVS (J) mice under fed and fasting (24 h) conditions. Quantitative data are shown as the means ± SD. Fed control mice values are set at 1.0 except PDK4 in skeletal muscle. For PDK4 in skeletal muscle, the fed JVS mice value is set at 1.0. * denotes $P < 0.05$ versus control mice under the respective feeding conditions. † denotes $P < 0.05$ versus the respective fed mice.

Fig. 3. Cellular distribution of PDK4 protein. (A) Levels of PDK2 and PDK4 proteins in homogenate (Ho) and mitochondria (Mit) fractions by a sucrose gradient procedure (see Methods) are shown. Each lane contains the same CS activity (1.5 mU) representing the same mitochondria amount. Components (E2, E3BP, E1α and E1β) of PDC are detected and identified by anti-PDC antibody (αPDC). "LDH activity" means the degree of contamination of cytosolic components. (B) Each fraction (Ho, 6 μg; S1, 3 μg; S2, 2 μg; S3, 1 μg; P4, 1 μg of protein/lane) was analyzed by the respective antibodies (αPDK2, αPDK4 and αPDC). Ho, homogenate; S1, 600 x g supernatant; S2, 20, 000 x g supernatant; S3, 100, 000 x g supernatant; P4, 100, 000 x g precipitation. (C) P4 fraction was fractionated with sucrose gradient centrifugation. PDK2, PDK4, PDC, KGDC (E2 and E3) and BCKDC (E2, E3 and E1α) were detected by the respective antibodies. The number corresponds to tube number fractionated from bottom to top of the gradient.

Fig. 4. Comparison of 2-DG uptake in tissues of control (open column) and JVS mice (gray column). Control and JVS mice under fed conditions were intraperitoneally injected

with [^{3}H]2-DG. At 30 min after injection, and the ^{3}H-radioactivity uptaken by the respective tissues, including the heart (H), skeletal muscle (SM), liver (L), brain (Br) and white adipose tissue (WAT) was measured (see Methods). Values are shown as the means ± SD (n = 6). * denotes $P < 0.05$ versus control mice.

Table 1

PDC active form and total activities under fed and starved conditions

	Fed		Starved (24 h)	
	Active	Total	Active	Total
	(mU/mg protein)			
Heart				
Control	9.8 ± 4.1 (4)	89.9 ± 7.8 (4)	0.5 ± 0.2 † (4)	60.0 ± 7.4 † (4)
JVS	6.3 ± 2.6 (4)	55.2 ± 9.2 * (4)	1.6 ± 0.6 *, † (4)	48.7 ± 11.4 (4)
Skeletal muscle				
Control	10.9 ± 4.5 (6)	12.5 ± 2.0 (4)	3.6 ± 0.7 † (6)	16.2 ± 5.1 (4)
JVS	11.5 ± 2.9 (4)	13.1 ± 4.4 (4)	2.3 ± 1.2 † (4)	16.5 ± 8.2 (4)

The number of mice used is shown in parenthesis. Values are expressed as mU of PDC activity over mg of total protein. Data are means ± SD. † denotes $P < 0.05$ versus control mice under respective feeding conditions. * denotes $P < 0.05$ versus respective fed mice.

Fig. 1.

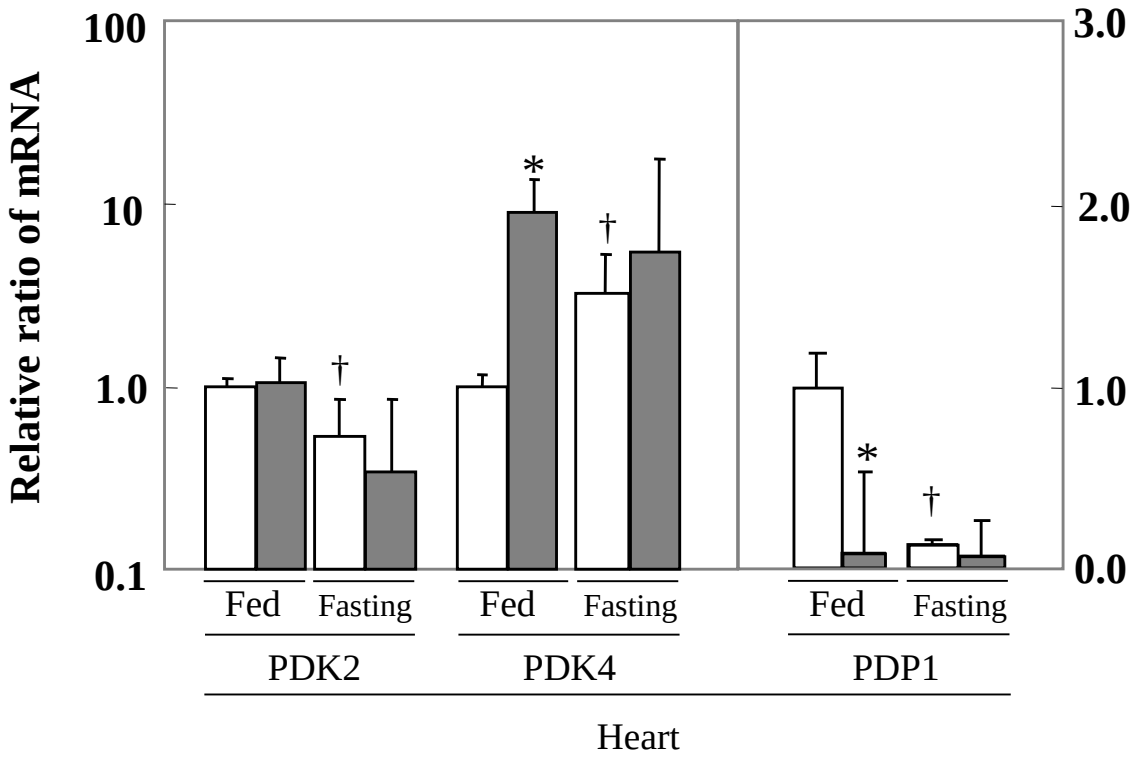
Relative ratio of mRNA
100
10
1.0
0.1
3.0
2.0
1.0
0.0
*
†
†
*
†
Fed
Fasting
Fed
Fasting
Fed
Fasting
PDK2
PDK4
PDP1
Heart

Fig. 2.

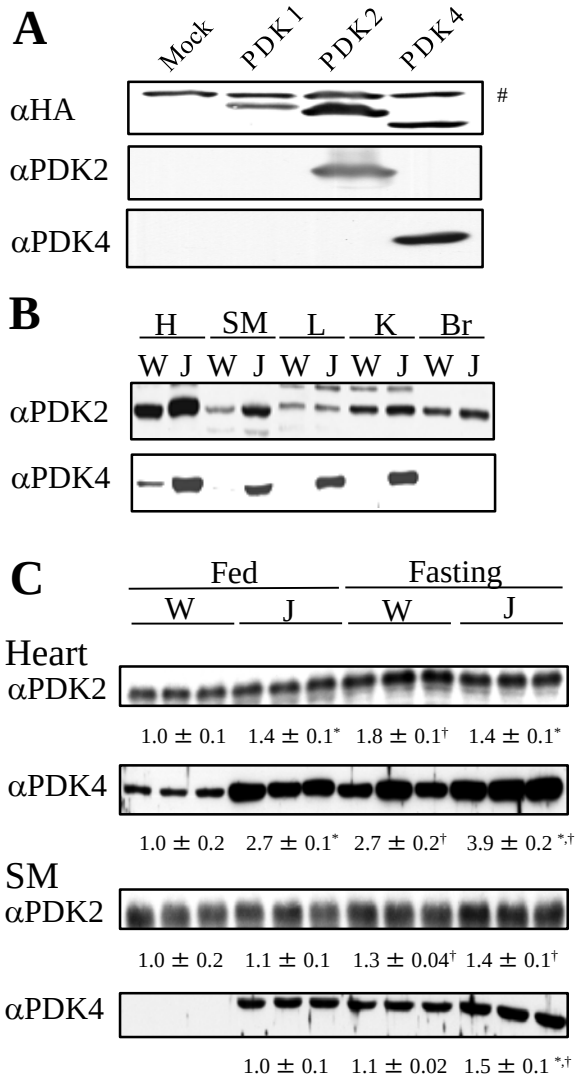
A
Mock
PDK1
PDK2
PDK4
αHA
#
αPDK2
αPDK4
B
H
SM
L
K
Br
W J W J W J W J W J
αPDK2
αPDK4
C
Fed
Fasting
W
J
W
J
Heart
αPDK2
1.0 ± 0.1
1.4 ± 0.1*
1.8 ± 0.1†
1.4 ± 0.1*
αPDK4
1.0 ± 0.2
2.7 ± 0.1*
2.7 ± 0.2†
3.9 ± 0.2 *,†
SM
αPDK2
1.0 ± 0.2
1.1 ± 0.1
1.3 ± 0.04†
1.4 ± 0.1†
αPDK4
1.0 ± 0.1
1.1 ± 0.02
1.5 ± 0.1 *,†

Fig. 3.

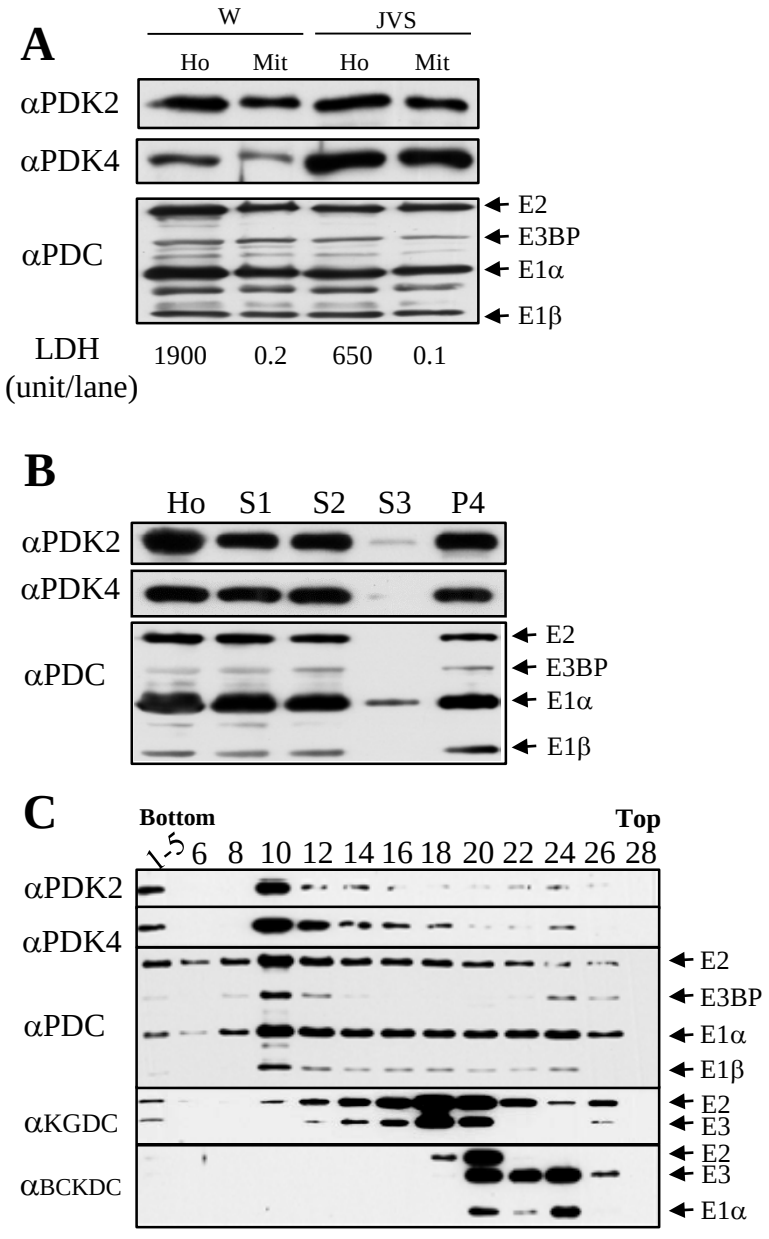
A
W
JVS
Ho
Mit
Ho
Mit
αPDK2
αPDK4
αPDC
E2
E3BP
E1α
E1β
LDH
(unit/lane)
1900
0.2
650
0.1
B
Ho
S1
S2
S3
P4
αPDK2
αPDK4
αPDC
E2
E3BP
E1α
E1β
C
Bottom
Top
1
5
6
8
10
12
14
16
18
20
22
24
26
28
αPDK2
αPDK4
αPDC
αKGDC
αBCKDC
E2
E3BP
E1α
E1β
E2
E3
E2
E3
E1α

Fig. 4.