Regulation of Pyruvate Dehydrogenase Kinase 4 in the Heart through Degradation by the Lon Protease in Response to Mitochondrial Substrate Availability

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Cardiac metabolic inflexibility is driven by robust up-regulation of pyruvate dehydrogenase kinase 4 (PDK4) and phosphorylation-dependent inhibition of pyruvate dehydrogenase (PDH) within a single day of feeding mice a high fat diet. In the current study, we have discovered that PDK4 is a short lived protein (t½ ~ 1 h) and is specifically degraded by the mitochondrial protease Lon. Lon does not rapidly degrade PDK1 and -2, indicating specificity toward the PDK isoform that is a potent modulator of metabolic flexibility. Moreover, PDK4 degradation appears regulated by dissociation from the PDH complex dependent on the respiratory state and energetic substrate availability of mouse heart mitochondria. Finally, we demonstrate that pharmacologic inhibition of PDK4 promotes PDK4 degradation in vitro and in vivo. These findings reveal a novel strategy to manipulate PDH activity by selectively targeting PDK4 content through dissociation and proteolysis.

Dynamic regulation of metabolism is required for cells to respond to nutrient availability and stress to support ATP production and anabolic processes. Misappropriated metabolic alterations can exert deleterious effects as evidenced in diabetes and in many cancers where the preference for a specific metabolic profile promotes the progression of disease (1, 2). The heart derives energy primarily from the oxidation of fatty acids. However, glucose utilization increases with enhanced availability and is essential for cardiac function, particularly in response to physiologic and pathophysiologic stress. Obesity and diabetes are characterized by heavy reliance of the heart on fatty acids for energy production and the inability to appropriately utilize glucose (1). Loss of metabolic flexibility is believed to underlie associated cardiovascular disease. In mouse models of diet-induced obesity, induction of cardiac metabolic inflexibility has long been attributed to insulin resistance and altered glucose transporter 4 expression and transport (1, 3). However, we have recently made the discovery that these events are preceded by diminished mitochondrial oxidation of glucose-derived pyruvate, occurring within the 1st day of high fat feeding (4).

The mitochondrial enzyme pyruvate dehydrogenase (PDH)2 commits glycolytically derived pyruvate for ATP production and is central to regulating the use of glucose relative to fatty acids for energy homeostasis. Cardiac PDH activity is regulated by various isoforms of pyruvate dehydrogenase kinase (PDK1, -2, and -4) and phosphatase (PDP1 and -2) with phosphorylation resulting in enzyme inhibition (5–7). We demonstrated that a selective increase in cardiac PDK4 expression and inhibition of PDH are responsible for the rapid diet-induced loss of mitochondrial pyruvate utilization and the ensuing development of insulin resistance in the heart (4). Given that PDK4-mediated inhibition of PDH is an initiating event in diet-induced metabolic inflexibility and PDH is a key site for control of glucose oxidation, it is critical to define the mechanism(s) that regulates PDK4 expression in response to alterations in fatty acid availability.

We have demonstrated that diet-induced increases in PDK4 content are rapidly reversible upon return of mice to a control diet (4). Proteolysis is therefore likely to play a vital role in regulation of PDK4 protein content, a possibility that has not previously been investigated. The goal of the current study was to identify the protease responsible for PDK4 degradation and molecular factors that regulate susceptibility to degradation. Our novel findings demonstrate that cardiac PDK4 is a short lived protein that is a specific substrate of the ATP-dependent mitochondrial protease Lon. Additionally, we determined that PDK4 degradation by Lon is predicated on the respiratory state of the mitochondria and the substrates utilized for energy production. Finally, we used this new knowledge to directly and specifically manipulate cardiomyocyte PDK4 content by altering the rate of degradation in vitro and in vivo. Our work reveals regulatory mechanisms that promote the selective degradation of PDK4, which can be exploited for treatment of diseases where PDK4 up-regulation is thought to play a role in pathogenesis.

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Results

PDK4 mRNA and Protein Content Is Rapidly Regulated in HL-1 Cells in Response to Fatty Acids—HL-1 cells, a mouse cardiac muscle cell line, retain a differentiated contracting phenotype in culture (8). To investigate the dynamic regulation of PDK4 expression, HL-1 cells were incubated with fatty acids (100 μM palmitate and 100 μM oleate). Fatty acids were readily consumed with ~50% depletion from the medium within 2 h (Fig. 1A). PDK4 mRNA and protein were up-regulated (Fig. 1B), and phosphorylation of PDH increased (Fig. 1C) within 2 h of fatty acid supplementation. PDK4 mRNA and protein subsequently returned to basal levels in concert with time-dependent exhaustion of fatty acids from the medium (Fig. 1B).

PDK4 Has a Short Half-life and Is Stabilized by Fatty Acids—Most mitochondrial proteins are relatively stable with half-lives on the order of days (9). However, PDK4 is likely short lived given that fatty acid-induced up-regulation of this protein is rapidly reversed upon consumption of fatty acids (Fig. 1, A and B). To estimate the half-life of PDK4, protein content was measured as a function of time in HL-1 cells incubated with cycloheximide (CHX) to inhibit protein translation. PDK4 exhibited a half-life of 1.1 ± 0.2 h (Fig. 1D and E). In contrast, PDK1 and -2 proteins remained unchanged with CHX treatment for 2 h (Fig. 1E). To determine whether fatty acids influence PDK4 degradation, cells were treated with CHX in the presence or absence of palmitate and oleate for 1.5 h. As shown in Fig. 1F, fatty acids suppress PDK4 degradation.

Mitochondrial Lon Protease Is Responsible for the Degradation of PDK4—PDK4 resides within the mitochondrial matrix. ClpXP and Lon, ATP-dependent proteases present in the mitochondrial matrix, were investigated as potentially responsible for PDK4 degradation. Treatment of HL-1 cells with the specific ClpXP inhibitor Z-LY-cmk did not alter PDK4 protein levels or the magnitude of PDK4 degradation in the presence of CHX (Fig. 2A). In contrast, MG262, an inhibitor of Lon and the proteasome, fully prevented PDK4 degradation (Fig. 2B). MG132, a specific inhibitor of the proteasome, had no effect on PDK4 degradation (Fig. 2B). PDK4 is therefore specifically degraded by Lon. Further support for this conclusion is provided using HL-1 cells in which Lon was knocked down with siRNA (Fig. 2C). The basal level of PDK4 protein was 2-fold...
Degradation was dependent on ATP and abolished in the presence of the Lon inhibitor MG262 (Fig. 3B). Inclusion of Ca^{2+}, an inhibitor of the PKDs (10) and activator of PDP1 (11), or dichloroacetic acid (DCA), a pan-specific inhibitor of PKD isoforms, resulted in an increase in the rate of PDK4 degradation (Fig. 3C). Finally, incubation of mitochondria with palmitoylcarnitine and malate prior to the degradation assay resulted in suppression of PDK4 degradation (Fig. 3C). Palmitoyl-CoA did not abrogate PDK4 degradation in the mitochondrial extract (Fig. 3D), indicating that fatty acid-induced reductions in PDK4 degradation (Figs. 1F and 3C) are not due to direct inhibition of Lon. Collectively, these results indicate that the activation state of PDH and/or PDK4 governs the susceptibility of PDK4 to ATP-dependent degradation by Lon.

**PDH-PDK4 Association Is Dynamically Regulated by the Activation State of PDH4—** PDK4 is an integral component of the PDH complex (12) bound to the lipoyl domain region of the transacetylase E2 subunit (L2) (13). Dissociation of PDK4 from PDH was investigated as a potential determinant of degradation. To estimate relative levels of complexed and uncomplexed PDK4, polyethylene glycol (PEG) was used to precipitate the PDH complex. We used this approach because, under our experimental conditions, immunoprecipitation with antibodies to PDK4 or the E1 or E2 (lipoic acid) subunits of PDH resulted in dissociation of the PDH complex or PDK4 and, in certain cases, limited immunoprecipitation efficiency. Although PEG precipitation experiments are nonspecific in nature, PDK4 not precipitated with the PDH complex is likely dissociated from other high molecular weight complexes.

Cardiac mitochondria were isolated from mice and incubated in the absence of respiratory substrate (state 1) or in the presence of pyruvate and malate (state 2) and following addition of ADP (state 3). Initiation of state 3 respiration is characterized by an increase in the rate of oxygen consumption accompanied by activation of PDH (Fig. 4A). Under each respiratory condition, the PDH complex was precipitated with 3% PEG, and the fraction of precipitated and soluble PDK4 was assessed (Fig. 4B). The fraction of uncomplexed PDK4 increased from 9% in the absence of substrate to 22 and 40% during state 2 and 3 respiration, respectively (Fig. 4C). Inclusion of DCA or Ca^{2+}, which are both activators of PDH and inhibitors of PDK4, resulted in greater dissociation of PDK4 (Fig. 4C).

When mitochondria were incubated with palmitoylcarnitine (PC), a respiratory substrate that leads to activation of PDK4 and inhibition of PDH, only 7% of PDK4 was free under all respiratory conditions (Fig. 4C). Therefore, conditions that favor PDK4 inhibition and PDH activation induce the release of PDK4 from the PDH complex or other high molecular weight complexes.

PDH catalysis, phosphorylation status, and PDK4 activity were each investigated as factors that trigger PDK4 dissociation and promote degradation. Incubation of mitochondria in the absence of respiratory substrate but in the presence of DCA or Ca^{2+} promoted dissociation of PDK4 from the high molecular weight fraction (Fig. 5A). PDK4 dissociation is therefore not dependent on the catalytic turnover of PDH. Furthermore, NaF...
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FIGURE 4. Metabolic regulation of PDK4 dissociation. Mitochondria were isolated from mouse heart. A, cardiac mitochondria (0.25 mg/ml) were incubated with 0.1 mM pyruvate and 1 mM malate as respiratory substrates. State 3 respiration was initiated upon addition of 0.25 mM ADP at 2 min. Oxygen consumption and PDH activity were measured. B, mitochondrial extracts were treated with 3% PEG to precipitate PDH and other high molecular weight proteins and complexes. The PEG pellet (P), but not the supernatant (S), contained both PDH (~65 kDa) and α-ketoglutarate dehydrogenase (KGDH) (~50 kDa) as assessed by Western blotting analysis using antibody to lipoic acid, a cofactor covalently bound to the E2 subunit of each complex. B and C, the amount of PDK4 protein that precipitated with PDH was determined following incubation of mitochondria (2.0 mg/ml) with respiratory substrates 0.8 mM pyruvate (Pyruvate) or 0.05 mM PC. Both incubations were supplemented with 2.0 mM malate, and 2.0 mM ADP was supplied to initiate state 3 respiration. Where indicated, Ca²⁺ (100 μM) or DCA (2 mM) was added to the incubation mixture. A 60-μl aliquot of mitochondrial protein was removed from the incubation for PEG precipitation during state 1 (no substrate), state 2 (ADP-independent), and state 3 (ADP-dependent) respiration. Western blotting analysis with antibody to PDK4 was used to quantify the relative levels of complexed and uncomplexed PDK4 in the pellet (P) and supernatant (S), respectively. These values were used to calculate the fraction of free (supernatant) relative to total (supernatant + pellet) PDK4. Each experiment was performed on five biological replicates (i.e. cardiac mitochondria isolated from n = 5 separate mice). The scatter plot data (C) are represented as the mean, and error bars represent ± S.D. with p values as follows: *<0.05; ***, <0.001.

Discussion

Unique findings from this study are 1) PDK4 has a short half-life and is specifically degraded by the Lon protease, 2) susceptibility to degradation is governed by the metabolic state of the mitochondria and promoted upon inhibition and dissociation of PDK4, and 3) pharmacologic inhibition of PDK4 in vivo induces selective degradation of PDK4. The rapid turnover of PDK4 is in striking contrast to the vast majority of heart mitochondrial proteins that have a median half-life of 17.2 days (9). Collectively, the mechanisms we have identified that regulate the rate of PDK4 degradation would result in stabilization of PDK4 and promote inhibition of PDH when fatty acid availability increases. Conversely, the susceptibility of PDK4 to degradation rises under conditions that require activation of PDK4 and utilization of glycolytically derived pyruvate. Non-transcriptional regulation of PDK4 protein may be an energetically favored mechanism to fine-tune cardiac metabolism during transient changes in lipid availability for which activation of large transcriptional networks would be unnecessary.

In the current study, we have identified PDK4 as a novel substrate of the mitochondrial protease Lon. PDK4 is now one of a few known natively folded Lon targets. For most known substrates of Lon, recognition and cleavage require exposure of hydrophobic amino acids (15). PDKs have hydrophobic C termini that bind to the lipoyl domain of the E2 subunit of PDH...
Thus, dissociation of PDK4 from the PDH complex may expose a hydrophobic region and confer susceptibility to Lon-mediated proteolysis. Properties that distinguish the short lived PDK4 from PDK1 and -2 that require investigation include lower binding affinity to the PDH complex and greater hydrophobic properties (17–19).

Altered expression of PDK4 has been implicated in the pathology of multiple diseases, including diet-induced cardiac metabolic inflexibility, ischemic heart disease, heart failure, type 2 diabetes, cancer, and Alzheimer’s disease (4, 20–28). Although development of specific inhibitors to PDK4 may help treat a variety of diseases, recent findings indicate that PDK4 (16). Thus, dissociation of PDK4 from the PDH complex may expose a hydrophobic region and confer susceptibility to Lon-mediated proteolysis. Properties that distinguish the short lived PDK4 from PDK1 and -2 that require investigation include lower binding affinity to the PDH complex and greater hydrophobic properties (17–19).

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can exert effects not solely dependent on kinase activity. PDK4 binds to and stabilizes the cAMP-response element-binding protein, resulting in mTORC1 activation and promotion of tumorigenesis (22). PDK4 also binds apoptosis-inducing factor, which promotes epithelial to mesenchymal transition in lung cancer cell lines (26). Our studies indicate that an inhibitor specific for PDK4 would achieve selective inhibition and degradation of PDK4 without impairing normal regulation of PDH complex by PDK1 and -2. Evidence we have provided on mechanisms and conditions that promote PDK4 degradation can also be exploited to increase PDK4 content in disease states where a reduction in PDK4 is associated with the proliferative capacity of certain cancers (23, 26).

Experimental Procedures

**Mice and Treatments—**Male C57BL/6N mice (Charles River Laboratories) at 6 weeks of age were utilized. For in vivo inhibition of PDK4, mice were administered DCA (70 mg/kg i.p. injection; Sigma 347795) and sacrificed 30 min postinjection. Mice were euthanized by cervical dislocation, and hearts were excised. All procedures were approved by the Oklahoma Medical Research Foundation Animal Care and Use Committee.

**HL-1 Cell Culture Maintenance and Treatment—**HL-1 cells (8) were maintained at 37 °C with 5% CO2 in Claycomb medium (Sigma 51800C) supplemented with 10% FBS (Sigma F2442, Batch 12J001), 2 mM L-glutamine (Sigma G7513), 0.1 mM non-epinephrine (Sigma A9037), 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma P4333). Experiments were conducted when cells were confluent and beating in glucose- and pyruvate-free DMEM supplemented with 10% FBS, 5 mM glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin. To treat with fatty acids, 2.0 mM palmitate (Sigma P9767) and 2.0 mM oleate (Sigma O7501) were prepared in a solution of 8% BSA (Sigma A8806, essentially fatty acid- and endotoxin-free) in DMEM. BSA-conjugated fatty acids were then diluted into the cell culture medium supplemented with 3 mM L-carnitine (Sigma C0283) to a final concentration of 200 µM total fatty acids (1:1 palmitate and oleate). The free fatty acid concentration in the medium was measured to assess uptake using the HR Series NEFA-HR(2) kit (Wako Diagnostics). The inhibitors used in the HL-1 experiments were 75 µM CHX (Sigma), 5 mM DCA (Sigma), 10 µM MG132 (Enzo Life Sciences), 10 µM MG262 (Boston Biochem), and 5 µM Z-LY-cmk (Bachem).

**siRNA Knockdown—**InterferIn siRNA Transfection Reagent (PolyPlus 409-01) was used to introduce siRNAs into HL-1 cardiomyocytes in DMEM free of FBS, penicillin, and streptomycin. Lon (Sigma siRNA identification number SASI Mm01 00082186) or control siRNA (Sigma SIC001) was added to a final concentration of 40 nM, and HL-1 cardiomyocytes were transfected for 24 h. Transfection medium was then replaced with complete Claycomb medium, and cells were harvested for analysis 48 h following transfection.

**Isolation of Cardiac Mitochondria—**Mitochondria were isolated from mouse heart as described (4) with the following modifications for HL-1 cells. Confluent T25 flasks were scraped in ice-cold isolation buffer (10 mM MOPS, 1.0 mM EDTA, 210 mM mannitol, and 70 mM sucrose, pH 7.4) supplemented with 20 mM NaF, 2 mM Na₃VO₄, protease inhibitor mixture (Roche Applied Science), and 0.01% digitonin. Cells were homogenized by pipetting, and mitochondria were isolated by differential centrifugation (4). The mitochondrial pellet was resuspended in isolation buffer containing 20 mM NaF and protease inhibitor mixture. Protein concentrations were determined using the bicinchoninic acid method (Pierce).

**Analysis of Mitochondrial Respiratory Function—**Isolated cardiac mitochondria were diluted to 0.25 mg/ml in 10 mM MOPS, 210 mM mannitol, 70 mM sucrose, and 5.0 mM K₂HPO₄ at pH 7.4 (respiratory buffer) containing respiratory substrates (pyruvate and malate) as indicated. State 3 respiration was initiated at 2.0 min by the addition of ADP at a final concentration of 0.25 mM. Rates of mitochondrial respiration were evaluated at room temperature using a Neofox oxygen chamber with a 175-µl volume (Instech Laboratories, Inc.) (4).

**Evaluation of PDH Activity—**As previously described (4), isolated cardiac mitochondria were diluted to 0.05 mg/ml in a buffer containing 25 mM MOPS and 0.05% Triton X-100 at pH 7.4. Solubilization of mitochondria with 0.05% Triton X-100 inhibits complex I of the respiratory chain, preventing consumption of NADH. PDH activity was measured spectrophotometrically (Agilent 8452A) as the rate of NAD⁺ reduction to NADH (340 nm, ε = 6,200 M⁻¹ cm⁻¹) upon addition of 2.5 mM pyruvate, 0.1 mM CoASH, 0.2 mM thiamine pyrophosphate, 1.0 mM NAD⁺, and 5.0 mM MgCl₂ at pH 7.4.

**Mitochondrial Degradation of PDK4—**Mouse cardiac mitochondria were incubated under the specified respiratory conditions. Mitochondria were then lysed by sonication and centrifuged at 15,000 × g for 10 min. The supernatant was removed and diluted in degradation buffer (30 mM Tris-HCl at pH 8, 10 mM MgCl₂, 1.3 mM DTT, and 10 mM ATP) to 1.75 mg of protein/ml. Samples were immediately transferred to 37 °C for 0–60 min. An aliquot of the incubation mixture was removed at the indicated times and analyzed by Western blotting for the rate of PDK4 disappearance.

**PEG Precipitation—**Cardiac mitochondria were diluted to 0.2 mg/ml in 25 mM MOPS, 2.7 mM EDTA, and 1% Triton X-100 following incubation under the indicated respiratory conditions. Solubilized mitochondria were then centrifuged at 16,000 × g for 10 min to remove the membrane fraction. The supernatant was brought to a pH of 6.45 with 10% acetic acid. Large molecular mass proteins were selectively precipitated by addition of PEG 6000 (Sigma) to a final concentration of 3% and mixed for 30 min at 4 °C (29). Precipitated protein was recovered by centrifugation (16,000 × g, 20 min, 4 °C) with each subunit of PDH found in the pellet as judged by mass spectrometry (not shown) and Western blotting analysis (Fig. 4). PDK4 that precipitated is likely primarily associated with the PDH complex, the main binding partner of PDK4. Nevertheless, we cannot rule out PDK4 dissociation from other high molecular weight complexes. Total protein precipitation from the supernatant was accomplished at a PEG concentration of 15%. PDK4 recovered from the second PEG precipitation is termed uncomplexed. Both pellets were dried and resuspended in equal volumes of loading buffer for Western blotting analysis. The percentage of uncomplexed PDK4 was calculated using the
formula (PK4 S/(PK4 S + PK4 P)) × 100 where S is supernatant and P is pellet.

Western Blotting Analysis—Mitochondria or precipitated protein was subjected to Western blotting analysis as described previously (4). Anti-phospho-PDH E1 (Ser293) was purchased from EMD Millipore (AP1062), anti-HSPO was from Santa Cruz Biotechnology (sc-1052), anti-PDK1 was from Enzo Life Sciences (ADI-KAP-PK112), and anti-PDK2 was from Abgent (AJ1598a). Rabbit polyclonal antibodies to lipoic acid (30), Lon, and PK4 were generated by Biosynthesis, Inc. Antisera were generated to the following peptide sequences: Lon, EPEAEN-KQKSRKLLKGGK; and PDK4, mixture of CIPSREPKNLAKE-KLA, DLVEFHEKSPEDQKALSE, and EFVDTLVKVRINHHNVT. Primary antibody binding was visualized using secondary antibodies conjugated to horseradish peroxidase (Pierce) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Densitometric analyses of Western blots were performed using ImageJ software in the linear range of band intensity. Assignment of molecular weights, as depicted in figures, was derived using amino acid sequences, expression, and regulation.

Quantitative RT-PCR—RNA was extracted using Tris buffer (Roche Applied Science 11667157001) from snap frozen heart tissue (~10 mg) or HL-1 cells. Quantitative PCR was performed as described previously (4). The transcript levels for target genes were normalized to three reference genes (Gapdh, Sdha, and Hspcb) determined to be unchanged between conditions. The primer pair used to quantify PK4 mRNA was as follows: forward, 5' - AGGGAGCTGGCTTTCTC-3'; and reverse, 5' - GAGGCTTCAATAACCGGTC3'.

Statistics—Data are presented as mean ± S.E. or S.D. as indicated. Statistical analyses were performed using the two-tailed Student’s t test and Bonferroni correction for multiple comparisons with p values denoted as follows: *, <0.05; **, <0.01; and ***, <0.001.

Author Contributions—C. C., C. S., L. I. S. M. K., and I. L. designed experiments. C. C., L. I. S., and M. K. analyzed data. I. L. contributed reagents. C. C., C. S., and L. I. S. conducted experiments. C. C. and L. I. S. wrote the manuscript.

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