PYRUVATE-DRIVEN OXIDATIVE PHOSPHORYLATION IS DOWNREGULATED IN SEPSIS-INDUCED CARDIOMYOPATHY: A STUDY OF MITOCHONDRIAL PROTEOME

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ABSTRACT—Background: Sepsis-induced cardiomyopathy (SIC) is a major contributing factor for morbidity and mortality in sepsis. Accumulative evidence has suggested that cardiac mitochondrial oxidative phosphorylation is attenuated in sepsis, but the underlying molecular mechanisms remain incompletely understood. Methods: Adult male mice of 9 to 12 weeks old were subjected to sham or cecal ligation and puncture procedure. Echocardiography in vivo and Langendorff-perfused hearts were used to assess cardiac function 24 h after the procedures. Unbiased proteomics analysis was performed to profile mitochondrial proteins in the hearts of both sham and SIC mice. Seahorse respirator technology was used to evaluate oxygen consumption in purified mitochondria. Results: Of the 665 mitochondrial proteins identified in the proteomics assay, 35 were altered in septic mice. The mitochondrial remodeling involved various energy metabolism pathways including subunits of the electron transport chain, fatty acid catabolism, and carbohydrate oxidative metabolism. We also identified a significant increase of pyruvate dehydrogenase (PDH) kinase 4 (PDK4) and inhibition of PDH activity in septic hearts. Furthermore, compared to sham mice, mitochondrial oxygen consumption of septic mice was significantly reduced when pyruvate was provided as a substrate. However, it was unchanged when PDH was bypassed by directly supplying the Complex I substrate NADH, or by using the Complex II substrate succinate, or using Complex IV substrate, or by providing the beta-oxidation substrate palmitoylcarnitine, neither of which require PDH for mitochondrial oxygen consumption. Conclusions: These data demonstrate a broad mitochondrial protein remodeling, PDH inactivation and impaired pyruvate-fueled oxidative phosphorylation during SIC, and provide a molecular framework for further exploration.

KEYWORDS—Bioenergetics, cardiomyopathy, citric acid cycle, mitochondria, oxidative phosphorylation, proteomics, sepsis

INTRODUCTION

Sepsis-induced cardiomyopathy (SIC) is a common complication of sepsis that results in circulatory failure and high mortality (1–4). While the etiology of SIC is complex and multifactorial, several studies have provided evidence that mitochondrial dysfunction plays a key role (3, 5). Clinically, there is a possible association between the degree of mitochondrial dysfunction, disease severity, and septic patient outcomes (3, 6). Yet, the molecular mechanisms underlying the mitochondrial dysfunction in the septic heart remain incompletely understood.

The mitochondria are the primary source of ATP to the working heart and encompass roughly 30 percent of the total volume of a heart muscle cell, the highest volume fraction of all mammalian cell types (7). Daily energy consumption by the heart is the highest of all the organs (8), requiring a constant and tightly regulated mitochondrial ATP production to meet these high energy demands (9, 10). Mitochondrial oxidative phosphorylation in the heart is fueled by fatty acid oxidation with additional contribution from the end product of carbohydrate (e.g., glucose) catabolism, pyruvate. Fatty acids are metabolized by beta-oxidation whereas pyruvate is decarboxylated to form acetyl-CoA by pyruvate dehydrogenase (PDH), the enzyme that links cytoplasmic glycolysis metabolic pathway to the mitochondrial citric acid cycle. The metabolism of fatty acids and pyruvate inside the mitochondrial matrix yields reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH2). NADH and FADH2 are the source of energy powering protons pumping out of the mitochondrial matrix by the electron transport chain
(ETC), which generate a large electrochemical proton gradient across the mitochondrial inner membrane. This gradient drives ATP production by ATP synthase (complex V) (11). The synthesized ATP then exits the mitochondrial matrix via adenine nucleotide translocase (ANT) and is used to fuel the production of contractile force, calcium signaling, and maintaining ionic gradients across sarcosomal and organellar membranes.

A number of studies demonstrate altered mitochondrial energy production and abnormal oxidative phosphorylation in the hearts of septic mice or mice treated with proinflammatory agents (3). These include decreases in oxygen consumption, decreased mitochondrial ATP synthesis due to impaired activities of several ETC complexes (12–15), and reduced adenine nucleotide translocator 1 (ANT1) (16). While these reports highlight the possible role of mitochondrial dysfunction in SIC, others report enhanced mitochondrial function in sepsis (17). Importantly, a recent study shows that restoring PDH activity in sepsis promotes mitochondrial bioenergetics in the splenocytes and hepatocytes and improves sepsis survival in mice (18).

In this study, we employed an unbiased proteomics approach to profile the mitochondrial protein expression in the heart. Among 665 detected proteins with a known mitochondrial localization or mitochondrial association, 35 varied significantly between healthy and septic hearts. These included those with key functions in glucose and fatty acid metabolism, as well as subunits of the electron transport chain. Of particular interest, we found an increase in PDH kinase isozyme 4 (PDK4), whose function is to phosphorylate pyruvate dehydrogenase (PDH) and inhibit its function (19, 20), and marked PDH inactivation in the septic hearts. Moreover, employing Seahorse respiratory assay, we found that mitochondrial oxygen consumption in the septic hearts was significantly reduced when pyruvate was provided as fuel. These findings support the hypothesis that mitochondrial protein remodeling such as PDK4 upregulation and PDH phosphorylation/inactivation may play an important role in cardiac metabolic dysfunction during SIC.

**METHODS**

**Animals**

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Adult male mice were between 9 and 12 weeks of age and weighed between 20 and 30 g. The animal protocols were approved by the Institutional Animal Care and Use Committee, University of Maryland School of Medicine (Approval Number: 0918001, Baltimore, MD). All animal experiments were performed in compliance with the relevant guidelines and regulations of the National Institutes of Health (Bethesda, MD) and the ARRIVE guidelines of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (London, EN).

**Cecal ligation and puncture**

A clinically relevant mouse model of sepsis was generated by cecal ligation and puncture (CLP) surgery as described previously with minor modifications (21–24). Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdominal cavity was opened in layers and the feces were gently migrated to fill the distal part of the cecum. The cecum was ligated approximately 1.0 cm from the tip and was punctured with an 18-gauge needle. A small droplet of saline (50 mL/kg body weight) was administered subcutaneously to both sham and CLP mice. Bupivacaine (3 mg/kg) and buprenorphine (0.1 mg/kg) were given postoperatively to manage pain.

**Echocardiography**

Echocardiography was performed in non-anesthetized mice as previously described (21).

**Langendorff measurements**

Mice were heparinized (1000 IU/kg, intraperitoneal) and anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Following confirmation of anesthesia, hearts were then excised and subjected to an ex vivo Langendorff perfusion model as previously described with minor modifications (21). A water-filled balloon was introduced into the left ventricle to record LV pressure (PowerLab, AD Instruments, Denver, CO). Hearts were paced at 420 bpm and left ventricular developed pressure (LVDp, left ventricular end systolic pressure) was recorded continuously for 30 min. LVDp was taken as an average of the pressure over those 30 min.

**ADP/ATP and ATP assay**

For the ADP/ATP ratio assay, hearts were snap-frozen and homogenized in ice-cold 2N perchloric acid (PCA) at 100 μL/10 mg tissue using a tissue tear for 5 s followed with Dounce homogenization for 25 to 30 passes. Samples were kept on ice for 45 min and centrifuged at 13,000 × g for 2 min at 4°C. One hundred microliters of supernatant was diluted with ATP assay buffer (Abcam, Cambridge, MA) and neutralized with 2 M KOH. Samples were then centrifuged again at 13,000 × g for 15 min at 4°C and supernatant was collected. Deproteinized and neutralized samples were then run using a commercially available ADP/ATP ratio assay kit from Abcam (Catalog No: ab65313) per manufacturer’s instructions. ADP/ATP ratio was calculated as described in the manufacturer’s protocol. For the ATP assay, mice hearts were snap frozen and homogenized in ice-cold 1% trichloroacetic acid and kept on ice for 45 min and centrifuged. Supernatants were neutralized with 1 M KOH. Deproteinized and neutralized samples were then assayed for ATP content as previously described (10).

**Isolation of cardiac mitochondria**

Mouse cardiac mitochondria were isolated as we previously described (10). It is worth noting that, in our protocol, we took additional steps that are not routinely used to increase purity but with the cost of lower yield. We pelleted the nonmitochondrial fractions at low-speed centrifugation of 600 × g three times. To pellet and isolate mitochondria, we centrifuged our samples at 3,200 × g instead of the more commonly used 10,000 × g. In addition, after we obtained the mitochondrial pellet, we repeated this step three more times to further discard any possible cytosolic or other soluble materials of the supernatant, whereas typical isolation protocols utilize only one cycle. As result, the mitochondrial preparations contain only trace amounts of GAPDH and SERCA (Suppl. Fig. 1, http://links.lww.com/SHK/B364), two proteins highly expressed in the cytoplasm and the sarcoplasmic reticulum, respectively, suggesting that the cardiac mitochondrial preparations in our study were highly pure and with minimal cytosolic or organellar contaminations. Subsequently, a BCA assay was used to quantify the concentration of isolated mitochondria (in mg mitochondrial protein per mL). The mitochondria were kept on ice and used for respiration measurements within 4h of isolation. The remaining mitochondria were stored at − 80°C for Western blots. Mitochondrial assay solution (MAS, 1X) was comprised of: 70 mM sucrose, 220 mM mannitol, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA, 0.2% (w/v) fatty acid-free BSA (pH = 7.2) (25). Drugs and substrates were prepared as described previously and diluted to the following final concentrations: 4 mM ADP, 2.5 μg/mL oligomycin, 4 μM FCCP, 4 μM antimycin A, 10 mM succinate, 2 mM malate, 2 μM rotenone, and 10 mM pyruvate (25).

**Seahorse respirator assay**

All Seahorse assays were performed using an XF24 plate as described previously with minor modifications (25). Based on the type of substrates used for the respiration assay, various amounts of isolated cardiac mitochondria in a volume of 50 μL, that is, 2.5 μg (succinate and rotenone), 5 μg (pyruvate and malate), or 7.5 μg (palmitoyl carnitine and malate) were delivered to each well in triplicate or quadruplicate technical replicates. Mitochondria were then centrifuged at 2000 × g for 20 min at 4°C in a swinging bucket microplate adapter to attach the mitochondria to the plate. After centrifugation, 625 μL of warm 1X MAS + substrate was added to each well. Either the complex I-linked substrates pyruvate (10 mM) and malate (2 mM), the complex II substrate succinate (10 mM) in the presence of the Complex I inhibitor rotenone (2.5 μM), or the beta-oxidation substrate palmitoyl carnitine (40 μM) and malate (1 mM) were given to the mitochondria in the 1X MAS. The plate was then
allowed to equilibrate at 37 °C for 10 min and subsequently transferred to the XF24 instrument for oxygen consumption rate (OCR) measurements. Three basal measurements were recorded for 3 min each with 1 min of mixing between each recording. Immediately following the last basal measurement, the following injection protocol was used:

1. Port A: 4 mM ADP
2. Port B: 2.5 μg/mL oligomycin
3. Port C: 4 μM FCCP
4. Port D: 4 μM antimycin A (AA).

Mix and measure times were 1 and 3 min, respectively, and drugs were injected immediately following the previous measurement. For the NADH-based respiration assay in which Complex IV activity was also specifically determined (26–28), 0.156 μg of isolated mitochondria were delivered to each well in triplicate or quadruplicate technical replicates. Three basal measurements were recorded for 2 min each with 2 min of mixing between each recording. Isolated mitochondria were then subjected to the following injection protocol:

1. Port A: 1 mM NADH, 40 μg/mL alamethicin, and 100 μM cytochrome C.
2. Port B: 2 μM rotenone.
3. Port C: 0.5 mM TMPD, 2 mM ascorbate, and 4 μM antimycin A.
4. Port D: 50 mM azide.

Wait, mix, and measure times were all 2 min long and drugs were injected immediately following the previous measurement.

### Proteomics

Mitochondria isolated from the hearts of sham or CLP mice were solubilized in 5% sodium deoxycholate and were washed, reduced, alkylated and trypsinolyzed in a filter as previously described (29, 30). Tryptic peptides were separated on a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 μm, 75 μm × 200 mm, Waters Corporation) over a 165-min linear acetonitrile gradient (3–40%) with 0.1% formic acid on a Waters nano-ACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap Fusion Lumos Trubrid mass spectrometer as described (31). Full scans were acquired at a resolution of 240,000, and precursors were selected for fragmentation by collision-induced dissociation (normalized collision energy at 35%) for a maximum 3-s cycle. Tandem mass spectra were searched against a UniProt database to yield a colored reaction product with an increase in absorbance at 450 nm at room temperature. Hearts were homogenized in ice-cold PBS containing protease and phosphatase inhibitors. Protein concentration was determined using a BCA protein assay and each sample was adjusted to 200 μg of protein. The assay was then performed to the manufacturer’s specifications in kinetic mode for 15 min at room temperature. The rate (ΔμOD/min) was then calculated by using the following formula:

$$Rate = \frac{ absorbance_{t} - absorbance_{0}}{time}$$

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad, La Jolla, CA). Unless stated otherwise, the distributions of the continuous variables were expressed as the mean ± SEM. Proteomic comparisons between the CLP mice and the sham were performed by FDR corrected, one-way ANOVA test and z-score. D’Agostino and Pearson (single comparison) or Kruskal–Wallis (multiple comparisons) normality tests were used to evaluate the normality of the data. If normal, the statistical significance of the difference between groups was measured by t test or two-way ANOVA with Sidak’s post hoc test for more than two groups. If the data did not pass the normality test, nonparametric tests (Mann–Whitney for single comparisons) were used to derive the P values. The null hypothesis was rejected for P < 0.05 with the two-tailed t test.

### RESULTS

#### Polymicrobial sepsis induces significant cardiac dysfunction and energy deficit

To model sepsis-induced cardiomyopathy (SIC), we subjected mice to cecal ligation and puncture (CLP) or sham laparotomy. Cardiac function was assessed at baseline prior to surgery and again at 6- and 24-h posturgery using echocardiography (Fig. 1A and B). We observed significantly decreased cardiac output, stroke volume, heart rate, LVIDd, and percent fractional shortening in the mice that underwent CLP surgery compared with sham (Fig. 1C). To rule out possible confounders associated with the load-dependent echocardiographic measurements, we isolated hearts from sham and CLP mice 24 h after procedures and subjected the mouse hearts to *ex vivo* Langendorff apparatus. Analysis of these hearts revealed a significant decrease in left ventricular developed pressure (LVDP) in the CLP group compared with sham (104.4 ± 12.9 vs. 54.3 ± 9.9 mmHg, sham vs. CLP, n = 3–4, P < 0.01) as well as in dp/dt maximum (3852 ± 416 vs. 2010 ± 341 mmHg/s) and minimum (−2.591 ± 293 vs. −1.376 ± 241 mmHg/s, sham vs. CLP n = 3–4, P < 0.01, Fig. 1D). To test if an energy deficit is present in the heart, we examined total ATP levels and the ADP/ATP ratio from sham and CLP hearts using commercially available assay kits. Interestingly, while total ATP levels remained the same between sham and CLP, CLP hearts exhibited a significantly higher ADP/ATP ratio (0.14 ± 0.02 vs. 0.26 ± 0.01, sham vs. CLP, P < 0.001) (Fig. 1E and F). These data indicate that CLP mice developed intrinsic cardiomyopathy and might possess myocardial energy deficit.

### Proteomics analysis reveals altered mitochondrial metabolic proteins in septic heart

One of the possible molecular mechanisms behind the contractile dysfunction during sepsis is the impairment of mitochondrial bioenergetics. We hypothesize that bioenergetic
FIG. 1. Cardiac dysfunction and ATP and ADP/ATP ratio in sepsis mice. (A) The timeline of experiments. A baseline echocardiography (Echo) was performed one day prior to surgical procedures. The next day, sham (laparotomy only) or CLP (laparotomy plus cecal ligation and puncture) surgery was performed and Echo used to assess cardiac function at 6 and 24 h postsurgery. After the 24 h Echo, animals were sacrificed, and mitochondria were isolated for subsequent experiments. (B) Representative Echo M-mode images showing LVIDd/LVIDs at baseline and 24 h after sham or CLP procedure. (C) Echo measurements at baseline and at 6 and 24 h after sham or CLP surgery. n = 6/group. (D) Ex vivo Langendorff-perfused hearts. Left ventricular (LV) developed pressure (LVDP) and rate of LVDP (dP/dT) were measured at 24 h after sham and CLP procedures. n = 3 to 4/group. (E–F) Total ATP and ADP/ATP ratio were measured 24 h after sham and CLP surgery in whole hearts. (E) Total ATP, n = 10 (sham) and 12 (CLP). (F) ADP/ATP ratio, n = 4 (sham) and 8 (CLP). Each error bar represents mean ± SEM. Data in (C) were analyzed using 2-way ANOVA with Sidak’s multiple comparisons test. Panels in (D–F) were analyzed using two-tailed t test. *P < 0.05, **P < 0.01, ***P < 0.001.
impairment and subsequent myocardial dysfunction during sepsis is associated with altered mitochondrial protein expression. To test this, cardiac mitochondria were isolated from sham mice and CLP mice exhibiting clear cardiac dysfunction (n = 6/group) and evaluated using liquid chromatography-tandem mass spectrometry. Of the 665 proteins with an established physical or functional mitochondrial association that were identified in the proteomics assay, 35 were found to be significantly altered between sham and CLP mitochondria (Fig. 2A). A complete list of the 35 proteins can be found in Table 1. Notably, these 35 differentially expressed mitochondria-associated proteins are reportedly involved in various bioenergetic functions such as pyruvate metabolism, fatty acid metabolism, lactate production, electron transport, and mitochondrial membrane integrity (Fig. 2B, Table 2). Pyruvate dehydrogenase kinase 4 (PDK4), a primary regulator of PDH, was significantly up-regulated in the mitochondrial fraction isolated from septic hearts. Pyruvate kinase (PK), the enzyme responsible for catalyzing the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to yield pyruvate and one molecule of ATP in the last step of glycolysis, was also upregulated.
Meanwhile, methylmalonyl-CoA epimerase (MCE), an enzyme involved in odd-chain fatty acid catabolism, was significantly down-regulated in sepsis heart mitochondria (Fig. 2C). In addition, we discovered up-regulation of two other proteins indirectly related to fatty acid metabolism, phytanoyl-CoA dioxygenase (PHYH), and carbonyl reductase [NADPH] 2 (CBR2). PHYH catalyzes the first step in the alpha-oxidation of phytanic acid while CBR2 metabolizes aldehydes and ketones derived from lipid peroxidation (Fig. 2C). Moreover, a subset of electron transport chain proteins, including NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8, a subunit of Complex I), cytochrome c oxidase 5B (COX5B, a subunit of Complex IV), cytochrome c oxidase copper chaperone (COX17, a nuclear-encoded protein essential for the assembly of Complex IV), were downregulated, while NADPH-cytochrome P450 reductase (P450R, an enzyme required for the transfer of electrons from NADPH to cytochrome P450), was up-regulated in the mitochondria of septic mice (Fig. 2D).

Increased PDK4 and phospho-PDH is associated with markedly attenuated PDH activity in septic heart

We performed qRT-PCR to test whether the observed increase in mitochondrial PDK4 protein expression in septic hearts was due to increased transcription. We found a 6.8-fold increase in PDK4 gene expression in the cardiac tissue of septic mice as compared to sham mice (Fig. 3A, \( P < 0.001 \)). In contrast, there was no difference in the gene expression of PDH E1alpha subunit between sham and sepsis hearts (Fig. 3B). In consistent with PDK4 gene expression, we also observed an

### Table 1. Proteomics analysis of cardiac mitochondrial proteins: 35 differentially expressed proteins between CLP and Sham mice

| # | Accession | Gene name | Expression ratio (CLP/Sham) |
|---|-----------|-----------|-----------------------------|
| Pyruvate metabolism | | | |
| 1 | P52480 | PKM | 1.706 |
| 2 | O70571 | PDK4 | 1.993 |
| Fatty acid metabolism | | | |
| 3 | Q9D115 | MCEE | 0.595 |
| 4 | P08074 | CBR2 | 1.704 |
| 5 | O35386 | PHYH | 1.921 |
| Lactate production | | | |
| 6 | P16125 | LDHB | 1.481 |
| 7 | A0A1B0GSX0 | LDHA | 1.668 |
| Electron transport | | | |
| 8 | Q9D6J5 | NDUFB8 | 0.592 |
| 9 | Q9D881 | GM11273 | 0.671 |
| 10 | P56394 | COX17 | 0.609 |
| 11 | P37040 | P450R | 1.497 |
| Mitochondrial membrane integrity | | | |
| 12 | Q7TNS2 | MINOS1 | 0.508 |
| 13 | Q3KNM2 | MARCH5 | 2.685 |
| Other | | | |
| 14 | A0A0A0MQF6 | GAPDH | 1.660 |
| 15 | P18242 | CTSD | 1.607 |
| 16 | P12242 | UCP-1 | 2.410 |
| 17 | Q6VE8 | SLC25A3 | 1.575 |
| 18 | P10605 | CT5 | 1.777 |
| 19 | Q9L56 | SFXN3 | 7.061 |
| 20 | Q9K2U2 | KRT5 | 2.413 |
| 21 | Q61335 | BCAP31 | 1.631 |
| 22 | Q8CPR5 | MRPL15 | 1.571 |
| 23 | Q9QU0 | RHOA | 1.964 |
| 24 | P54797 | TANGO2 | 1.514 |
| 25 | A6ZIA | ALDOA | 1.549 |
| 26 | O90023 | TPP1 | 1.457 |
| 27 | Q9DM5 | SLC25A19 | 0.645 |
| 28 | Q6VE96 | SLC35F6 | 1.577 |
| 29 | Q3JMX0 | RPP14 | 0.453 |
| 30 | Q8R5Q6 | CDDCS8 | 1.907 |
| 31 | Q6YW4 | HDH3 | 0.602 |
| 32 | Q3FY8 | TRMT10C | 1.503 |
| 33 | Q8VDK1 | NIT1 | 0.571 |
| 34 | Q24L1 | LETMD1 | 0.656 |
| 35 | Q9DCJ7 | AURKAI1 | 1.610 |

### Table 2. Primer sequences for qRT-PCR

| Gene Primer sequence | Primer sequence |
|----------------------|-----------------|
| PDHE1a Forward 5'GGG ACG TCT GTT GAG AGA GC-3' | Reverse 5'TGT GTC CAT GGT AGC AGG TGT AA-3' |
| PDK4 Forward 5'CAG CTG GTG AAG AGC TGG TA-3' | Reverse 5'CTG CTT TGG TGG ATT TGC GTA-3' |
increase in PDK4 protein expression in mitochondria isolated from septic hearts as compared with that of sham hearts (Fig. 3C and D, *P < 0.05). Since the primary role of PDK4 is to phosphorylate the PDH E1 subunit, which is known to inhibit PDH enzyme activity, we performed Western blots using whole heart tissue from sham and CLP mice and found a significant increase in phosphorylated PDH at serine-293 in septic hearts (Fig. 3E and F). We then examined whether these molecular modifications were associated with altered PDH activities. We discovered that the myocardial tissues derived...
from septic mice had markedly lower PDH activity than that of sham mice (6.5 ± 4.2 vs. 27.4 ± 2.4 AmO2/min, Sham vs. CLP, n = 5, P < 0.0001) (Fig. 3G and H).

**Cardiac mitochondria of septic mice exhibited a reduction in ADP-stimulated and uncoupled oxygen consumption rate when fueled with pyruvate/malate**

We assessed mitochondrial oxygen consumption rate (OCR) using a Seahorse Extracellular Flux assay. To distinguish between pyruvate-driven respiration that depends on both PDH activity and Complex I and respiration that depends on Complex I only, isolated mitochondria were either fueled with pyruvate/malate as substrates or were permeabilized to directly deliver the Complex I substrate NADH. The exogenous electron donor TMPD was tested downstream of NADH addition to assess Complex IV activity (Fig. 4A and B). We found that compared to mitochondria from sham mice, mitochondria isolated from septic hearts exhibited a similar level of basal respiration (Fig. 4C), but significantly lower state 3 respiration (ADP-stimulated) (110.90 ± 5.65 vs. 89.79 ± 4.31 pmol O2/min/μg, sham vs. CLP, n = 12, P < 0.01) (Fig. 4D) and state 3 uncoupled respiration (226.20 ± 24.37 vs. 160.90 ± 21.57 pmol O2/min/μg, sham vs. CLP, n = 12, P < 0.05) (Fig. 4E) when fueled with pyruvate/malate. States IV respiration was unchanged (Fig. 4F). Importantly, NADH-fueled OCR (Complex I) and TMPD-fueled OCR (Complex IV) each exceeded the maximal pyruvate/malate-driven OCR by ≥10-fold and displayed no differences between sham and CLP (Fig. 4G and H). Moreover, succinate (in the presence of rotenone) and palmitoylcarnitine were also used as substrates to assess Complex II-dependent respiration and beta oxidation-driven respiration, respectively. No OCR differences between septic and sham mitochondria were observed when mitochondrial respirations were fueled by these substrates (Fig. 4I and J). The decreased OCR in the presence of pyruvate/malate but not Complex I, Complex II, Complex IV, or beta-oxidation substrates suggests that CLP mitochondria may have reduced capacity to metabolize pyruvate leading to the reduced stage III respiration. In addition, the dramatically lower uncoupled OCR by mitochondria oxidizing pyruvate/malate compared with Complex I (NADH) or IV (TMPO) substrates is consistent with the notion that NADH supply from PDH and other mitochondrial matrix dehydrogenase enzymes is rate-limiting for mitochondrial respiration in the heart.

**DISCUSSION**

In a mouse model of polymicrobial sepsis, we demonstrated sepsis-induced cardiomyopathy with significant contractile dysfunction as illustrated by echocardiographic measurements in vivo and in isolated Langendorff-perfused hearts ex vivo. Energy production and/or demand may also be reduced as septic hearts exhibited a higher ADP/ATP ratio. To profile mitochondrial proteins in the heart, we carried out a quantitative proteomics analysis on cardiac mitochondria purified from septic and sham mice, which revealed differential expressions of 35 mitochondrial proteins between sepsis and sham mice. One of the mitochondrial proteins, PDK4, the primary kinase that phosphorylates and inactivates PDH, was upregulated whereas PDH activity was markedly decreased in the septic hearts. Moreover, oxygen consumption rate (OCR) by mitochondria from the septic hearts were significantly decreased as compared with the sham controls when respiration was fueled by pyruvate and malate. Importantly, OCR did not differ between the two groups when NADH and TMPD were used to directly drive Complex I and IV respiration, respectively, or when succinate was used to test respiration that is dependent on Complex II and downstream components of the ETC, or when the beta-oxidation substrate palmitoylcarnitine was used. These data suggest that in murine sepsis, cardiac dysfunction is associated with marked mitochondrial PDH inhibition and impaired pyruvate-fueled oxidative respiration in the heart.

The proteomic analysis identified a total of 1,135 proteins, 665 of which appeared to be mitochondria or metabolism-associated proteins. To enhance rigor in our study, we only focused on proteins that were highly abundant in both the sham and the CLP groups. Of these proteins, we focused on mitochondrial proteins that increase/decrease by approximately ~1.5 fold with such effects having statistical significance of a P < 0.05. Furthermore, we would like to stress that no conclusion was made solely based on the proteomic data. Any protein of interest highlighted by the proteomic analysis was further tested by another method. It should be noted that while proteins belonging to all four ETC complexes were detected in both sham and CLP cardiac mitochondria, only Complex I and Complex IV subunits exhibited differential expression between sham and CLP mice. In addition, we found other proteins involved in pyruvate metabolism, fatty acid metabolism, and electron transport to be perturbed in mitochondria isolated from the septic hearts. Notably, enzymes traditionally involved in beta-oxidation did not show altered expression. Therefore, the functional significance of the proteomic findings of these enzymes, that is, methylmalonyl-CoA epimerase (MCE), phytanoyl-CoA dioxygenase (PHYH), and carbonyl reductase [NADPH] 2 (CBR2) remains to be investigated. MCE catalyzes fatty acids with odd-length carbon chains. PHYH is a peroxisome-associated protein involved in alpha-oxidation of fatty acids, and CBR2 metabolizes aldehydes and ketones derived from lipid peroxidation (35). Lipid peroxidation occurs when free radicals gain electrons from lipids, causing the formation of fatty acid radicals. As sepsis is known to generate high levels of reactive oxygen species (ROS) (36, 37), it is speculated that these mitochondria have fatty acid radicals, resulting in an increased need for CBR2-dependent detoxification. Moreover, we did not see reduction in the adenine nucleotide translocator (ANT1) in cardiac mitochondria of CLP mice, in contrast to a report that TNFα-treated mice have myocardial inflammation and reduced ANT1 expression (16). Overall, the proteomics analysis revealed several metabolic pathways that were disturbed in septic mitochondria, providing the molecular basis for future explorations of the mechanisms of mitochondrial dysfunction in sepsis-induced cardiomyopathy.

One of the interesting findings in this study was the phosphorylation and inhibition of cardiac PDH activity during sepsis. PDH activity is an overall rate-limiting step in oxidative phosphorylation that is driven by pyruvate. Therefore, the reduction in OCR driven by pyruvate/malate may be explained by the observed PDK4 upregulation and PDH inhibition in...
FIG. 4. Oxygen consumption by cardiac mitochondria of sham and CLP mice. Heart mitochondria were isolated from sham or CLP mice 24 h after surgery. (A and B) Representative tracings of mitochondrial OCR in triplicate with pyruvate/malate as substrates (A) or subjected to the electron donor NADH-based assay to test Complex I and IV respiration (B). For the pyruvate/malate-fueled respiration assay, oxygen consumption was measured before and after the sequential additions of ADP, oligomycin, FCCP, and antimycin A. For the NADH-based assay, oxygen consumption was measured before and after the sequential additions of NADH+alamethicin+cytochrome C, rotenone, TMPD+ascorbate+antimycin A, and sodium azide. Protocol for all injections is described in the section of Materials and Methods. (C–F) OCR for sham and CLP mitochondria during basal (basal—antimycin A), state 3 (ADP—antimycin A), state 4 (oligomycin—antimycin A), and state 3 uncoupled (state 3u, FCCP—antimycin A) respiration in the presence of pyruvate and malate. n = 12 mice/group. (G and H) OCR for sham and CLP mitochondria during Complex I (NADH—rotenone, G) and Complex IV (TMPD—azide, H)-dependent respiration. n = 5 (sham) and 6 (CLP). (I–J) OCR for sham CLP mitochondria during basal, state 3, state 4, and state 3 uncoupled in the presence of succinate and rotenone, or palmitoylcarnitine and malate. n = 6 to 9 mice/group. Each dot represents respiration data from a single mouse mitochondrial preparation measured in technical replicates. Each error bar represents mean ± SEM. For (C and J), data was analyzed using two-tailed t test unless the data did not pass the normality test. In that case, data was analyzed by Mann–Whitney test. *P < 0.05, **P < 0.01. (AA, antimycin A; ADP, adenosine diphosphate; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; OCR, oxygen consumption rate; oligo, oligomycin; Rot, rotenone; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine).
septic heart. It should be pointed out that similar change in PDH activity has been reported in other cells/tissues in sepsis, such as skeletal muscle, peripheral blood mononuclear cells (38–42), and splenocytes/hepatocytes (18). Moreover, McCall and co-workers reported that inhibition of PDK using dichloroacetate reversed PDH phosphorylation and improved mitochondrial respiration in hepatocytes and splenocytes (18). Using a chronic sepsis model induced by intra-abdominal placement of a fecal-agar pellet containing *E. coli* and *B. fragilis* in rats, Vary and co-workers demonstrated PDH inactivation in skeletal muscle after 5 days. Others demonstrate PDK4 upregulation and PDH inhibition following LPS treatment in cells and in animal models (43, 44). In a swine model, LPS infusion led to elevated levels of PDK4 transcript in the myocardium (45), although PDH activity was not assessed in the study. Expanding from these early studies, we identified a functional association between PDH inactivation and significant and specific reduction in pyruvate/malate-fueled OCR in the heart and in the absence of Complex I, II, and VI dysfunction. Moreover, and somewhat surprisingly, we did not find any difference between sham and septic mitochondria in the fatty acid palmitoylcarnitine-fueled mitochondrial respiration.

Currently, there are few therapies specifically designed to target myocardial bioenergetics in SIC. The standard clinical management of sepsis and septic shock includes antibiotics, fluid resuscitation, vasopressors, and positive inotropes among others (46–48). Other investigative therapies include preventative approaches such as the use of 3-hydroxy-3-methylglutaryl coenzyme A reductase or statins, which have anti-inflammatory and antioxidative properties. Statins in particular, represent a novel therapeutic approach for sepsis-induced cardiomyopathy. Surprisingly, despite the proteomics data demonstrating a significant decrease in NADH dehydrogenase 1 beta subcomplex subunit 8 (NDUFB8), a Complex I accessory subunit, we did not find any functional impairment with the NADH-driven Complex I respiration in mitochondria isolated from the septic hearts. Several previous studies suggested that Complex I activity is reduced during sepsis or endotoxemia in the heart and in the brain, including studies that utilized isolated cardiac mitochondria (15, 49, 50). However, these studies reported decreased enzymatic activity of Complex I, but did not assess the functional ability of the mitochondria to utilize Complex I-linked substrates that depend on the activity of the citric acid cycle dehydrogenases such as PDH. Another study into the role of Parkin during endotoxemia did investigate mitochondrial function in the heart after single-dose intraperitoneal injection of either PBS or LPS in WT or park2−/− mice (51). This study found reduced Complex I activity in the hearts of WT mice 12 h after LPS injection, which recovered at 48 h post-LPS treatment.

There were several limitations in the study. For one, we did not verify the changes of all 35 of the differentially expressed proteins that were found in our proteomics data by Western blot, as we elected to focus more on PDH and mitochondrial oxidative phosphorylation function. We also did not test all of the differentially expressed proteins found by the proteomics and there were likely some changes to low abundance proteins that were below the sensitivity of detection. Therefore, there are probably additional metabolically related proteins that are involved in the cardiac energy deficit during sepsis. It must be stressed that molecular remodeling, as identified by mass spec, even when confirmed by other molecular tests such as Western blot, does not necessarily imply functional impairment. Additionally, substrate availability in the

![Fig. 5. Molecular remodeling of cardiac mitochondrial in murine sepsis.](image-url) Proteomic analysis of mitochondria from hearts with sepsis-induced cardiomyopathy revealed several mitochondria-specific proteins with altered expression including PDK4 and the electron transport chain proteins NDUFB8, COX17, COX5B, and P450R. Increase in PDK4 expression leads to phosphorylation and inactivation of PDH, the enzyme converting pyruvate, NAD+, and coenzyme A into acetyl-CoA, CO2, and NADH. Diminished PDH activity limits the overall capacity of the mitochondria to utilize the metabolic substrate pyruvate resulting in a lower oxygen consumption rate as shown by the schematic above. These molecular modifications may provide insight into how the mitochondria is linked to the development of sepsis-induced cardiomyopathy (CBR2, carbonyl reductase [NADPH] 2; COX17, cytochrome c oxidase copper chaperone; COX5B, cytochrome c oxidase 5B; LDH, lactate dehydrogenase; MCE, methylmalonyl-CoA epimerase; NDUFB8, NADH ubiquinone oxireductase subunit B8; OCR, oxygen consumption rate; P450R, NADPH-cytochrome P450 reductase; PDK4, pyruvate dehydrogenase kinase 4; PHYH, Phytanoyl-CoA 2-Hydroxylase; p-PDH, phospho-pyruvate dehydrogenase).
mitochondrial respiration experiments is unlimited whereas bioavailability in vivo during sepsis may be compromised, especially with a decrease in energy demand in the septic heart, for example, slower heart rate and lower core temperature. More comprehensive functional testing of the metabolic pathways identified by the proteomics data will be pursued in our future studies.

In summary, by employing a proteomics approach, we identified 35 mitochondrial or metabolism-associated proteins that displayed differential expression in cardiac mitochondria from mice with SIC when compared with sham control (Fig. 5). Notably, PDK4 was upregulated at both mRNA and protein levels, along with an increase in the phosphorylated PDH and marked reduction in PDH activity in septic heart homogenate. These molecular remodeling events were associated with reduced mitochondrial capability to utilize pyruvate and likely contributed to decreased oxidative phosphorylation in the septic heart. These findings provide a molecular framework to better understanding the etiology of sepsis and sepsis-induced cardiomyopathy.

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