Toll-Like Receptor-Mediated Inflammatory Signaling Reprograms Cardiac Energy Metabolism by Repressing Peroxisome Proliferator-Activated Receptor γ Coactivator-1 Signaling

Joel Schilling, MD, PhD; Ling Lai, MD, PhD; Nandakumar Sambandam, PhD; Courtney E. Dey, BA; Teresa C. Leone, BS; Daniel P. Kelly, MD

Background—Currently, there are no specific therapies available to treat cardiac dysfunction caused by sepsis and other chronic inflammatory conditions. Activation of toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) is an early event in Gram-negative bacterial sepsis, triggering a robust inflammatory response and changes in metabolism. Peroxisome proliferator–activated receptor-γ coactivator-1 (PGC-1) α and β serve as critical physiological regulators of energy metabolic gene expression in heart.

Methods and Results—Injection of mice with LPS triggered a myocardial fuel switch similar to that of the failing heart: reduced mitochondrial substrate flux and myocyte lipid accumulation. The LPS-induced metabolic changes were associated with diminished ventricular function and suppression of the genes encoding PGC-1α and β, known transcriptional regulators of mitochondrial function. This cascade of events required TLR4 and nuclear factor-κB activation. Restoration of PGC-1β expression in cardiac myocytes in culture and in vivo in mice reversed the gene regulatory, metabolic, and functional derangements triggered by LPS. Interestingly, the effects of PGC-1β overexpression were independent of the upstream inflammatory response, highlighting the potential utility of modulating downstream metabolic derangements in cardiac myocytes as a novel strategy to prevent or treat sepsis-induced heart failure.

Conclusions—LPS triggers cardiac energy metabolic reprogramming through suppression of PGC-1 coactivators in the cardiac myocyte. Reactivation of PGC-1β expression can reverse the metabolic and functional derangements caused by LPS-TLR4 activation, identifying the PGC-1 axis as a candidate therapeutic target for sepsis-induced heart failure. (Circ Heart Fail. 2011;4:474-482.)

Key Words: sepsis ■ toll-like receptor 4 ■ heart failure ■ inflammation ■ metabolism

Bacterial sepsis is a common clinical disorder that accounts for more than 200 000 deaths in the United States annually. The septic syndrome is characterized by robust systemic inflammation and end-organ dysfunction. The heart is affected in at least 50% of patients with sepsis, and its presence portends a worse prognosis. Alterations in myocardial metabolism are known to occur during sepsis; however, the links between inflammatory signaling and myocardial fuel and energy metabolism remain unclear. To date, attempts to modulate myocardial metabolism in the setting of sepsis have not been reported.

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The heart’s high-capacity, ATP-generating mitochondrial system is under the control of a gene regulatory circuit. Members of the nuclear receptor transcription factor superfamily, including peroxisome proliferator–activated receptors (PPARs) and the estrogen-related receptors (ERRs), control the expression of many genes involved in mitochondrial energy transduction pathways including fatty acid β-oxidation (FAO), the electron transport complex, and oxidative phosphorylation. The nuclear respiratory factors [NRF-1 (Nrf1) and NRF-2 (Gabpa)] coordinate the transcription of nuclear and mitochondrial genes involved in electron transport and mitochondrial DNA replication. The expression of several of these genes has been shown to be suppressed by inflammation at the transcriptional level; however, the upstream signals and functional relevance of this response are unclear.

The activity of the PPARs, ERRs, and NRFs is orchestrated by PGC-1α and β (Ppargc1a and b), inducible, cardiac-enriched transcriptional coactivators. Targeted
disruption of the PGC-1α and PGC-1β genes in mice causes mitochondrial dysfunction and heart failure, evidence that deactivation of this pathway can lead to myocyte contractile dysfunction.12,13 The PGC-1 gene regulatory circuit is also deactivated in heart failure through mechanisms that have not been defined.14 Inflammatory signaling has been reported to both augment or suppress the expression or activity of the PGC-1 coactivators.8,15–17 The interpretation of these studies is further confounded by the lack of corroborative functional and metabolic data. Thus, questions remain regarding the regulation and physiological impact of the PGC-1 coactivators during times of inflammatory stress.

The inflammatory response and left ventricular dysfunction induced by lipopolysaccharides (LPS) is mediated in large part by the host molecule toll-like receptor 4 (TLR4).18 Interestingly, TLR4 signaling is also important in other cardiovascular diseases including ischemia-reperfusion injury and cardiac hypertrophy.19–21 Activation of TLR4 leads to the nuclear translocation of nuclear factor (NF)-κB, which drives the expression of inflammatory cytokines.22 We hypothesized that TLR4-mediated inflammation could serve as the upstream signal that modulates myocardial energy metabolism in sepsis. Our data demonstrated that the acute administration of LPS recapitulated aspects of the metabolic reprogramming known to occur in the failing heart, including downregulation of PGC-1α and PGC-1β gene expression, leading to reduced mitochondrial substrate flux and lipid accumulation. The suppression of PGC-1 gene expression was shown to occur through a TLR4 and NF-κB–dependent mechanism. Restoration of PGC-1 coactivator activity in cardiac myocytes during acute inflammation reversed LPS-induced alterations in cardiac fuel metabolism in vitro and in vivo.

**Methods**

**Animal Experiments and Generation**

Adult (age, 8 to 12 weeks) male C57BL/6 mice were obtained from Jackson Laboratory. TLR4 and TLR2 knockout mice (C57BL/6 background) were purchased from Oriental BioService, Inc (Japan). Adult mice (8 to 16 weeks of age) were used for experiments. To generate the TRE–PGC-1β line, Pparc1β cdNA was cloned into the pTRE-2 vector (Clontech), linearized with AatII and Asel, and injected into fertilized mouse eggs. Positive founders were identified by transgene-specific polymerase chain reaction, using the following primers: forward 5'-TCCGTGAGTTACCATTCTGAT-3' and reverse 5'-TCCGTGAGTTACCATTCTGAT-3'. Reverse 5'-GAGGAGACATGGGTTCTCAAC-3'. The TRE–PGC-1β mice were crossed to the MHC-rtTA line to produce TRE–PGC-1β MHC-rtTA mice. TRE–PGC-1β MHC-rtTA mice (FVB background) were fed a high-capacity mitochondrial system, we next assessed the effect of LPS on the myocardial expression of the PGC-1α and PGC-1β genes. LPS injection led to a rapid downregulation of PGC-1α and PGC-1β mRNA levels, with significant diminished (Figure 2A). PGC-1α and PGC-1β protein levels were also significantly diminished (Figure 2A).

**LPS-Triggered Activation of Inflammatory Pathways in the Cardiac Myocyte Results in Suppression of the PGC-1 Gene Regulatory Cascade**

Given the regulatory role of PGC-1 coactivators in maintaining a high-capacity mitochondrial system, we next assessed the effect of LPS on the myocardial expression of the PGC-1α and PGC-1β genes. LPS injection led to a rapid downregulation of PGC-1α and PGC-1β mRNA levels, with PGC-1β being the most dramatically affected (reduced >90%; Figure 2A). PGC-1α protein levels were also significantly diminished (Figure 2A).

PGC-1 coactivators modulate the expression of metabolic transcription factors [PPARα, ERRα, NRF1 (Nrf1), NRF2 (Gabpa)] and their downstream gene targets involved in FAO [MCPT-1 (Cpt1b), MCAD (Acadm), LCAD (Acadl), VLCAD (Acadvl)] and mitochondrial function [COX4 (Cox4t1), ATP synthase β (Atp5b)] (Figure 2B and 2C and online-only Data Supplement Figure 1A). Similar to the effects on PGC-1 coactivators, the expression of all of these metabolic genes...
was reduced at the mRNA level after LPS injection. In addition, expression of the PGC-1 target gene encoding the glucose transporter GLUT4 (Slc2a4) was also decreased by LPS instillation. Other genes involved in the regulation of FAO such as PPAR/β/H9251 and the myocardial FA transporter CD36 (Cd36) were not differentially expressed in the inflamed myocardium (online-only Data Supplement Figure 1A). These gene expression results are similar to those observed in the hearts of PGC-1/H9251 and PGC-1/H9252 double knockout mice,13 supporting the concept that LPS-mediated inflammation leads to rapid deactivation of the PGC-1 metabolic gene regulatory circuit.

To assess whether the observed effects of LPS occur in cardiac myocytes independent of systemic alterations such as ventricular dysfunction, LPS stimulation experiments were conducted with primary neonatal rat cardiac myocytes in culture. The effects of LPS on metabolic gene expression paralleled that seen in the heart in vivo, including reduced expression of genes encoding PGC-1α, PGC-1β, PPARα, ERRα, MCAD, and GLUT4 (Figure 3A, 3B, and 3C). To determine whether the LPS-mediated suppression of PGC-1α gene expression involved transcriptional mechanisms, a mouse PGC-1α promoter luciferase reporter (PGC.Luc.2112) was used for transient transfection studies in cardiac myocytes. LPS reduced the activity of PGC.Luc.2112 by approximately 50% but activated an NF-κB responsive control reporter (Figure 3D).

AMP-activated protein kinase (AMPK) is an energy-sensing kinase known to stimulate expression of the PGC-1α
TLR4 and NF-κB Are Required for LPS-Mediated Suppression of PGC-1α and PGC-1β Gene Expression

To address whether the changes in PGC-1 coactivator expression were dependent on functional TLR4, LPS challenge experiments were conducted with wild-type, TLR4−/−, and TLR2−/− mice, followed by assessment of gene expression and myocardial triglyceride levels at 6 hours. The absence of TLR4 abolished LPS-induced downregulation of PGC-1α and significantly blunted the downregulation of PGC-1β (Figure 4A). The increase in serum and myocardial lipids was also attenuated in mice lacking TLR4 (Figure 4A). The increase in serum and myocardial lipids was dependent on functional TLR4, LPS challenge arguing that AMPK is not upstream of the observed metabolic changes (online-only Data Supplement Figure 1B). To determine the role of TNFα and IL-6 in the suppression of PGC-1α, we used inhibitory antibodies to block their biological activity. Neither inhibition of TNFα or IL-6 prevented the downregulation of PGC-1α by LPS (online-only Data Supplement Figure 2A and 2B). Cardiac myocytes were also pretreated with the iNOS inhibitor L-NIL which, despite preventing LPS-induced NO production, had no affect on PGC-1α gene expression (online-only Data Supplement Figure 2C). Together, the data argue that TLR4 and NF-κB are required for the suppression of PGC-1α expression by LPS; however, this effect is largely independent of TNF, IL-6, and NO.

Cardiac-Specific Induction of PGC-1β Reverses LPS-Triggered Suppression of Myocardial Fatty Acid Oxidation and Ventricular Dysfunction

To further explore the role of PGC-1 coactivators in the cardiometabolic effects of LPS, we assessed the impact of reactivating PGC-1β expression in cardiac myocytes during TLR4-mediated inflammatory stress. To force PGC-1β expression in LPS-treated cardiac myocytes, the cells were infected with an adenovirus expressing PGC-1β and green fluorescent protein (GFP) (Ad-PGC-1β) or GFP alone (Ad-GFP) for 24 hours before LPS treatment. Ad-PGC-1β-infected cardiac myocytes were resistant to LPS-mediated suppression of PPARα, ERRα, and FAO gene expression (Figure 5). Importantly, LPS-mediated suppression of PGC-1α expression was preserved in Ad-PGC-1β–infected cells, indicating that the upstream events involved in suppressing PGC-1 gene expression were unaffected by PGC-1β overexpression. Similarly, inflammatory cytokine expression was not attenuated; rather, it was augmented in Ad-PGC-1β–infected cells.

To extend the rescue studies to an in vivo system, we used a doxycycline-inducible, dual-transgenic mouse system to force expression of PGC-1β specifically in cardiac myocytes (TRE–PGC-1βMHCrtTA mice23). For these experiments, nontransgenic and TRE–PGC-1βMHCrtTA mice were given doxycycline-containing chow 48 hours before PBS or LPS injection. In hearts of nontransgenic mice, LPS led to profound downregulation of PGC-1β mRNA levels. As expected, myocardial expression of PGC-1β in TRE–PGC-1βMHCrtTA mice was induced ~6- to 7-fold and the expression level was unaffected by LPS. Myocardial PGC-1α gene expression was equally suppressed by LPS treatment in both nontransgenic and TRE–PGC-1βMHCrtTA mice (Figure 6). Interestingly, the suppression of PGC-1 gene targets by inflammatory and metabolic events induced by LPS in the myocardium.

NF-κB is a key downstream target of TLR4 signaling. To determine the importance of NF-κB activation in this system, cardiac myocytes were pretreated with a chemical inhibitor of IκB kinase, Bay 11–7085. Compared with DMSO-treated control mice, Bay 11–7085 completely blocked the suppression of PGC-1α and PGC-1β expression after LPS stimulation (Figure 4C). As expected, Bay 11–7085 also decreased the expression of the NF-κB–regulated cytokines, TNFα, and IL-6 (Figure 4C). TNFα, IL-6, and iNOS are induced by LPS in a TLR4 and NF-κB–dependent manner and have all been implicated in sepsis-induced cardiodepression.27,28 To determine the role of TNFα and IL-6 in the suppression of PGC-1α, we used inhibitory antibodies to block their biological activity. Neither inhibition of TNFα or IL-6 prevented the downregulation of PGC-1α by LPS (online-only Data Supplement Figure 2A and 2B). Cardiac myocytes were also pretreated with the iNOS inhibitor L-NIL which, despite preventing LPS-induced NO production, had no affect on PGC-1α gene expression (online-only Data Supplement Figure 2C). Together, the data argue that TLR4 and NF-κB are required for the suppression of PGC-1α expression by LPS; however, this effect is largely independent of TNF, IL-6, and NO.
LPS was largely reversed by induction of PGC-1β (Figure 6). Notably, LPS-mediated induction of inflammatory gene expression (TNFα, iNOS, and IL-6) occurred to comparable or greater levels in TRE–PGC-1βMHCrtTA mice relative to nontransgenic animals (online-only Data Supplement Figure 3). These results provide further evidence that downregulation of the PGC-1 circuit is a critical metabolic event downstream of TLR4-mediated inflammation. To assess the metabolic consequences of PGC-1β induction in the setting of LPS stimulation, myocardial palmitate oxidation rates were determined in hearts isolated from nontransgenic and TRE–PGC-1βMHCrtTA mice treated with LPS or PBS. In nontransgenic mice, we observed the expected decline in FAO rates with LPS treatment. In contrast, TRE–PGC-1βMHCrtTA animals were protected from this effect (Figure 7A). The LPS-mediated increase in myocardial triglyceride accumulation was also reduced by ~50% in TRE–PGC-1βMHCrtTA mice. Importantly, serum lipid levels were similar in nontransgenic and TRE–PGC-1βMHCrtTA mice, demonstrating that the reduction in myocardial triglyceride is not a consequence of altered lipid delivery to the heart (Figure 7B).

To investigate whether cardiac myocyte expression of PGC-1β modulates cardiac contractile function during LPS-induced inflammatory stress, echocardiography was performed on nontransgenic and TRE–PGC-1βMHCrtTA mice after LPS injection. PGC-1β overexpression by itself did not produce structural or functional cardiac abnormalities as illustrated in the PBS control mice (Table and Figure 8A and 8B). Nontransgenic mice (FVB) injected with LPS had a significant decline in cardiac function, as evidenced by a reduction in left ventricular fractional shortening (52.6±0.6% to 24.0±1.3%). These results were similar to those seen with C57BL/6 mice (Figure 1B), except the baseline anesthesia effects on cardiac function are more pronounced in the C57BL/6 strain. In striking contrast, TRE–PGC-1βMHCrtTA mice exhibited only a modest decrease in cardiac function compared with PBS-injected littermates (50.8±1.2% versus 37.9±0.9%; Table and Figure 8A and 8B).
In addition to enhancing mitochondrial function and metabolism, PGC-1 coactivators can induce the expression of reactive oxygen species (ROS) scavengers. The generation of ROS in the myocardium occurs rapidly after LPS administration and is linked to myocardial dysfunction. To assess the effects of PGC-1 overexpression on myocardial oxidative stress after LPS injection, we determined tissue levels of reduced glutathione (GSH) in nontransgenic and TRE–PGC-1α mice after PBS or LPS injection. As shown in Figure 8C, LPS caused a drop in myocardial GSH levels 6 hours after injection, consistent with increased levels of oxidative stress. In contrast, LPS did not induce a decrease in myocardial GSH levels in transgenic animals. The mRNA expression of ROS scavengers known to be influenced by PGC-1 revealed that the mitochondrial enzyme superoxide dismutase (SOD)2 was upregulated in TRE–PGC-1α mice (Figure 8D). These data suggest that in addition to their metabolic effects, the PGC-1 coactivators probably influence oxidative stress levels in the myocardium.

Discussion
Sepsis is a common clinical disease characterized by widespread inflammation and end-organ dysfunction. The heart is frequently involved in this syndrome and contributes to the likelihood of death. At present, there are no effective therapies to improve myocardial function during sepsis. TLR4 plays a key role in initiating the inflammatory response during Gram-negative bacteremia by stimulating the production of NF-κB–dependent cytokines. Attempts to treat sepsis by preventing upstream activation of TLR4 and/or cytokine signaling have been largely unsuccessful in patients. Therefore, identification of potential targets downstream of the inflammatory response is of significant interest. Sepsis is known to diminish oxidative metabolism in the heart and other tissues, suggesting that targeting inflammatory-metabolic “cross-talk” could improve outcome in this syndrome. Herein, we describe a cascade of events that links TLR4 activation to myocardial metabolism through downregulation of PGC-1 coactivators.

Previous studies have reported variable effects of LPS on cardiac fuel and energy metabolism. Using an isolated working heart system, we demonstrated that LPS led to a consistent decrease in palmitate oxidation rates compared with controls. Together with an increase in circulating lipids, the reduction in myocardial capacity for fat oxidation promotes the development of myocardial steatosis, a pathological finding also observed in the hearts of humans with sepsis. Consistent with these findings, TLR4 was recently shown to mediate a similar fuel utilization shift in human primary skeletal muscle cells in culture.

The presence of increased myocardial lipids normally triggers a compensatory increase in the transcriptional activity of PPARα and ERRα and the expression of their coactivators PGC-1α and PGC-1β, leading to an increase in capacity for FAO. However, in our system, LPS diminished myocardial FAO rates despite an elevation in plasma lipids, suggesting a direct effect of inflammation on the machinery that regulates FAO enzyme expression. To determine the mechanism by which LPS alters myocardial metabolism, we initially focused our investigation on PGC-1α and PGC-1β, given their ability to “boost” the activity of several transcription factors that regulate mitochondrial FAO. Previous studies have demonstrated both upregulation and downregulation of PGC-1 coactivator expression during inflammation, which
The suppression of PGC-1α gene expression by LPS was found to occur at least in part at the transcriptional level. Moreover, blocking the TLR4:NF-κB axis prevented the downregulation of PGC-1 coactivator expression by LPS. We excluded AMPK, a known activator of PGC-1α,10 as well as TNFα, IL-6, and NO as mediators of LPS-induced suppression of PGC-1 gene expression. Whether NF-κB directly mediates the suppression of PGC-1 gene transcription, as has been recently proposed,38 or acts indirectly through the production of another molecule is an area of active investigation.

The conflicting data surrounding the effects of sepsis and LPS on the expression and activity of PGC-1 coactivators have been confounded in part by the lack of corroborative functional and physiological studies. To determine whether the suppression of myocardial FAO triggered by LPS:TLR4 signaling requires deactivation of the PGC-1 axis, we used PGC-1β gain-of-function strategies. Forced PGC-1β expression in cardiac myocytes in culture and in vivo reversed the suppression of FAO/mitochondrial gene expression induced by LPS. Importantly, reactivation of PGC-1β did not alter the induction of upstream inflammatory mediators or the downregulation of the related coactivator, PGC-1α, after LPS challenge. To validate the physiological relevance of these gene expression changes, we demonstrated that forced PGC-1β expression protected mice from LPS-induced suppression of mitochondrial FAO. Consistent with this finding, myocyte triglyceride accumulation was significantly decreased after LPS stimulation in TRE–PGC-1βMHCrtTA compared with nontransgenic mice despite similar increases in the levels of circulating lipids. That the LPS-mediated downregu-

Table. Echocardiographic Measurements in Nontransgenic and TRE–PGC-1βMHCrtTA Mice at Baseline and With LPS

<table>
<thead>
<tr>
<th></th>
<th>NTG (PBS)</th>
<th>NTG (LPS)</th>
<th>PGC-1β (PBS)</th>
<th>PGC-1β (LPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>393±17</td>
<td>463±11*</td>
<td>385±23</td>
<td>468±9*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.57±0.14</td>
<td>3.65±0.09</td>
<td>3.65±0.09</td>
<td>3.52±0.06</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>1.69±0.06</td>
<td>2.78±0.10*</td>
<td>1.80±0.07</td>
<td>2.19±0.06*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>52.5±0.6</td>
<td>24.0±1.4*</td>
<td>50.8±1.2</td>
<td>37.9±0.9†</td>
</tr>
<tr>
<td>LPWT, mm</td>
<td>0.91±0.03</td>
<td>0.96±0.02</td>
<td>0.94±0.03</td>
<td>0.94±0.03</td>
</tr>
</tbody>
</table>

NTG indicates nontransgenic mice; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; FS, fractional shortening; LPWT, left ventricular posterior wall thickness; and PGC-1β, TRE–PGC-1βMHCrtTA mice.

*P<0.05, PBS versus LPS.
†P<0.05 NTG versus PGC-1β (2-way ANOVA analysis).
lation of PGC-1α is still observed in TRE–PGC-1αMHCrtTA mice argues that the rescue of metabolism by PGC-1β is not a secondary consequence of disrupting upstream cytokine signaling or improving cardiac function. Although the use of a nonphysiological overexpression system to deliver PGC-1β is a limitation of this model, it serves as an important proof-of-concept experiment for future efforts toward targeting this gene regulatory circuit as a therapeutic strategy.

Myocardial dysfunction is a common and important finding in patients with sepsis, yet there are currently no therapies directed toward improving cardiac function in this syndrome. In the present study, we demonstrate that cardiac myocyte–specific PGC-1β overexpression leads to significantly improved cardiac function after LPS treatment. The mechanistic basis for this finding probably is multifactorial but may relate to the normalization of metabolic derangements and/or a reduction in oxidative stress levels. Previous studies have demonstrated that LPS injection rapidly triggers ROS generation in the myocardium and PGC-1 coactivators regulate the expression of several ROS scavenging enzymes. Consistent with this, we observed an increase in ROS with LPS that was reversed by PGC-1β overexpression. Gene expression analysis revealed that of the PGC-1 ROS scavenger targets, only SOD2 was significantly modulated with PGC-1β overexpression. Thus, PGC-1 appears to influence oxidative stress generation in addition to modulating metabolism in response to inflammatory stress. These findings, combined with evidence that “recognition” of PGC-1 expression in human patients with sepsis is predictive of survival, suggest that strategies to target PGC-1 expression/activity in cardiac myocytes may prove to be a novel therapeutic approach for the prevention and treatment of sepsis-induced cardiac dysfunction. Moreover, the suppression of PGC-1 expression by inflammatory signals may be a common theme in more diverse forms of heart failure.

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Disclosures
Dr Kelly received consulting fees for his advisory board relationships with Eli Lilly and Company and Johnson & Johnson.

References


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**CLINICAL PERSPECTIVE**

Sepsis syndrome is a significant cause of morbidity and mortality worldwide. Myocardial dysfunction is a common feature of this condition and is a predictor of worse clinical outcome. At present, there are no specific therapeutic targets for the cardiodepressant effects of systemic inflammation. Attempts to disrupt the early inflammatory phase of sepsis have been unsuccessful at improving outcomes. Therefore, targeting pathological pathways downstream of the inflammatory cascade, such as cellular metabolic derangements, has the potential to be a more fruitful approach. Previous studies have suggested that organ failure in sepsis may be related to dysfunctional mitochondrial metabolism, leading to a reduction in energy production and an increase in reactive oxygen species generation. We demonstrate that systemic inflammation activated by bacterial lipopolysaccharides reduces oxidative metabolism in the heart. This metabolic shift is associated with deactivation of the peroxisome proliferator–activated receptor-γ coactivator-1 (PGC-1) transcriptional circuit, a key regulator of mitochondrial function. By using both cell culture and in vivo approaches, we demonstrate that reactivation of PGC-1 during acute inflammation restores the expression of its downstream gene targets, reverses the decline in fatty acid oxidation, reduces oxidative stress, and improves cardiac function. These data suggest that the PGC-1 axis represents a novel therapeutic target for sepsis-induced cardiac dysfunction.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Protein Analysis. Crude nuclear extracts were prepared by sonicating tissue in ice cold buffer containing 0.255M sucrose, 1mM EDTA, 20mM Tris pH 7.4 and protease inhibitors followed by centrifugation at 5000 rpm for 20 minutes. The pellet resuspended in 1 ml of TNET buffer (1% Triton-X 100, 150 mM NaCl, 50 mM Tris pH 7.4, 2 mM EDTA) followed by a second centrifugation. The pellet was resuspended in 250 μl of RIPA buffer (1% NP40, 0.5% SDS, 0.1M PMSF, and protease inhibitors in PBS) and protein was quantified using BCA method (Thermo Scientific). 100 μg of nuclear extract was loaded on a 7.5% polyacrylamide gel. Western blot was performed with a PGC-1α antibody (EMD ST1202), 1:1000 dilution using Supersignal Femto reagent (Thermo Scientific). Total cellular protein lysates were prepared from NRCMs to assess PGC-1β expression or AMPK phosphorlyation using RIPA buffer (as above). 25 μg of total protein was run on a 7.5% polyacrylamide gel and the membrane was probed with the indicated antibodies. The following antibodies were used: AMPKα and phopho (Thr172) AMPKα (Cell Signaling – 2603,2535 ) and STAT3 and phosphor-STAT3 (Santa Cruz Biotechnology – sc-7179, sc-8059). The PGC-1β polyclonal antibody was a kind gift from A. Kralli.

Animal Experiments and Generation. Adult (8-12 weeks) male C57BL/6 mice were obtained from Jackson Laboratory (cat#000664). TLR4 and TLR2 knockout mice in the C57BL/6 background were purchased from Oriental BioService, Inc (Japan). Adult mice (8-16 weeks of age) were used for experiments. To generate the TRE-PGC1β line, Ppargc1β cDNA was cloned into the pTRE-2 vector (Clontech), linearized with AatII and AseI, and injected into fertilized mouse eggs (FVB). Positive founders were identified by transgene-specific PCR using the following primers: For 5’-
TCCGTGAGTTACCATTCTGAT-3', Rev 5'-GAGGAGACAATGGTTGTCAAC-3'. The TRE-PGC-1β mice were crossed to the MHC-rtTA\(^1\) line to produce TRE-PGC-1β\(^{MHC-rtTA}\) mice. TRE-PGC-1β\(^{MHC-rtTA}\) mice were fed chow containing doxycycline (Research Diets C11300-200i) adlib for 2 days. Mice (10-16 weeks of age) were given intraperitoneal injections of 150 µg (FVB) or 200 µg (C57BL/6) of \(E.\text{coli}\) 0111:B4 LPS (Sigma – L2630) resuspended in PBS or an equal volume of PBS alone. Injections were performed in the morning and food was removed from all animals immediately after the injection to control for the anorexia induced by LPS.

All animal experiments were conducted in strict accordance with NIH guidelines for humane treatment of animals and were reviewed by the Animal Studies Committee of Washington University School of Medicine.

**Isolated working heart preparation.** Isolated working heart perfusions were performed as previously described\(^2,3\) using adult male mice (10-14 weeks of age) 12 hours after the injection of LPS or PBS (all mice were fasted from the time of injection to control for the anorexia inducing effects of LPS). Hearts were subsequently perfused with Krebs-Henseleit solution containing 5 mM glucose, 100µU/ml insulin, and 0.4 mM palmitate.

**Plasma and Tissue chemistry.** Plasma was harvested from the inferior vena cava at the time of mouse sacrifice, transferred to a 1.5 ml microfuge tube, and centrifuged at 5000 rpm for 5 min. The supernatant or myocardial tissue sample was snap frozen in liquid nitrogen and stored at -80° C until analysis. Myocardial and plasma FFA and TAG concentrations were determined using a colorimetric assay by the Clinical Nutrition Research Unit (CNRU) at Washington University School of Medicine.

**Histology.** A mid-ventricular cross-sectional slice of myocardium was immersed in Tissue-Tek OCT Compound (Sakura Finetek USA Inc.) and frozen in liquid nitrogen. Tissue was sectioned and stained
with oil red O to detect intracellular neutral lipid accumulation. Histology was performed by the Digestive Diseases research Core Center (DDRCC) at Washington University School of Medicine.

**RNA Analysis and Real Time Quantitative PCR (qRT-PCR).** Total cellular RNA isolation from mouse cardiac ventricles and NRCMs was performed using the RNazol B method and the Qiagen RNeasy kit, respectively, according to the manufacturer’s instructions. RNA was reverse transcribed (Applied Biosystems) using random hexamer priming. Real time qRT-PCR was performed using SYBR green reagent (Applied Biosystems) and mouse/rat-specific primers (Supplemental Table 1) on the ABI Prism 7500 Sequence Detection system. Signal intensity was normalized to 36B4.

**Echocardiographic Studies.** Transthoracic M-mode and 2-dimensional echocardiography was performed on mice anesthetized with inhaled isofluorane in the Washington University Cardiovascular Phenotyping Core using a Visual Sonics 770 echocardiography system. Methods for measurements and chamber size using M-mode have been described.4

**Cell Culture.** NRCMs were prepared from 1-day old Sprague-Dawley rats (Charles River) using the Worthington Neonatal Cardiomyocyte Isolation kit per manufacture’s protocol. The cells were subsequently treated with 5 µg/ml LPS or PBS for 18 hours. Adenovirus overexpressing PGC-1β (Ad-PGC-1β) was generated by cloning mouse PGC-1β cDNA in to the AdTrack-CMV vector at HindIII and Xba1 sites followed by recombination with pAd-Easy as previously described.5 NRCMs were infected with adenovirus expressing GFP (Ad-GFP) or PGC-1β +GFP (Ad-PGC-1β) on day 1 after plating and on day 2 were treated with 5 µg/ml of LPS or PBS for 18 h after which mRNA was isolated. The NFκB inhibitory compound BAY 11-7085 (SABiosciences) was used a concentration of 6.25 µMol/liter in DMSO and added to NRCMs 1 h prior to LPS or PBS stimulation. CLI95 (InvivoGen-trl-cli95) was used a concentration of 10 µMol/liter in DMSO and added to NRCMs 1 h prior to LPS or PBS.
stimulation. L-NIL (Cayman Chemical – 80310) was used at a concentration of 10 \( \mu \text{Mol/liter} \) and added to NRCMs 30 min prior to LPS or PBS stimulation. Nitrate levels in the supernatant were determined using the Griess reaction. Briefly, equal volumes of cell culture supernatants were mixed with a 1:1 solution of 1.32% sulfanilamide in 60 % acetic acid and 0.1% N-1-naphthyl-ethylenediamine-HCL in water in a 96-well plate. The absorbance at 546 nm was determined for each sample and reported concentrations were based on standard curve for sodium nitrate. For inhibitory antibody experiments a neutralizing antibodies against rat TNF-\( \alpha \) (R and D Systems, AF-510-NA) or IL-6 (R and D Systems, AF506) were used a 5 \( \mu \text{g/ml} \) and 2 \( \mu \text{g/ml} \), respectively. Rat TNF-\( \alpha \) (R and Systems, 510-RT-010) was used at a dose of 10 ng/ml. The promoter luciferase construct for murine PGC-1\( \alpha \) (PGC.Luc.2112) has been described previously.\(^6\) The NF\( \kappa \)B luciferase construct was created by cloning 3 consensus NF-\( \kappa \)B binding sites (CGGGACTTTCC) into the BamH1 upstream of a luciferase reporter gene using tk-LUC vector. NRCMs were transfected at the time of plating using standard calcium-phosphate coprecipitation method.\(^7\) Day 2 after transfection, cells were stimulated with LPS or PBS for an additional 24 h. Luciferase activity was directly measured and normalized to SV40 renilla signal.

**Statistical Analysis.** All results are expressed as means +/- SEM and analyzed using students t-test or 2 way ANOVA as appropriate. \( P \leq 0.05 \) was considered statistically significant.
**Supplemental Table 1.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer Sequences (5’-3’)</th>
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| m36B4     | For - ATCCCTGACGCAACCCGCGTT GA  
             | Rev - TGACATCTGGCTTGGAGCACCAGTTC |
| mPGC-1α   | For - CGGAAATCATATATCACAACGAG  
             | Rev - TGAGGACCCGCTGAGAATGTTTG |
| mPGC-1β   | For - TCCAGAAGTCAGCCAAGCTCCT  
             | Rev - CTCGAATACAAGCCTTACGC |
| mERRα     | For - AAGAGTACGTCGCTCGTCTGCT  
             | Rev - CTCGACATCTTTCAATG |
| mERRβ/δ   | For - TCACCAGCAATTTCCACGCA  
             | Rev - AACCAGGCCCTTCTCTGCTT |
| mMCPT1    | For - GAAAATGATCAACACAAAGTTAT  
             | Rev - ATGCGCCACACATCAGA |
| mLCAD     | For - TTTCCGGAGAGTTGTAAGGA  
             | Rev - CTCGTTGCCACGCTTTCAC |
| mVLCAD    | For - ATCTAGCCTCCAGGAGCTTCT  
             | Rev - TTCTGGCTTGTTCCAGAAGTCAT |
| mNRF-1    | For - ACATTTGCGTAGCTGCTCACAGAA  
             | Rev - TGCGTCGTCTGGAATGCTGCT |
| mNRF-2    | For - GAGTCATCTGAGCCTTCTGCAA  
             | Rev - GTGTAAGCAGGACGACTGA |
| mCOXIV    | For - CTGTAAGCGGAGGCACCTGA  
             | Rev - TGACATGGCCACACATCAG |
| m/r ATPsynthase B | For - CTTGGCGGCTGCAA  
                     | Rev - CACAGCCTCAGCATCTTCAA |
| mCitrate Synthase | For - CAAGCAGCAACATGGGAAGA  
                       | Rev - GTCGAGATCAAGAACCAGAAAGTCT |
| mGLUT4    | For - ATCATACTGGAACATCGTGGAGG  
             | Rev - GTCAAGACACATCAGCCACAG |
| mGLUT1    | For - TCGTTGCCATCTCCTATTGTGC  
             | Rev - ACAGGAAGCAGACACTGAGCAG |
| mPDK4     | For - CCGCTGTCCATGAGAACCA  
             | Rev - GCAAGAAAAAGCAAAGAGCAGT |
| mCD36     | For - CACACATTGGAATCTTTTCC  
             | Rev - TCTCTTTAAGTGCTGATTTGCAAC |
| mTNFa     | For - CATCTTTCATCAACAAAATTCGAGTGACAA  
             | Rev - TGGGAGTAGACACAAGGTGACACCC |
| mL-6      | For - ACAGGCCCTCCTACTTCCAAAC  
             | Rev - ATCATCGTGTTTGCTACATCAAC |
| mNOS2 (iNOS)  | For - ACATCGACGCCCCGCTCCACAAGT  
                              | Rev - CAGAGGATGAGCTTGGCATC |
| mMCP1     | For - GCCCTGAGCAAGATCATGTA  
             | Rev - CCTACTCTATGGGATCTGAGCTTGC |
| mIFNβ     | For - GACGGAGAAGTAGCTGCAAGAGGTT  
             | Rev - AGTTCAATCAGGAAGAGTACAAAC |
| rPGC-1α   | For - GTGCAACGCAACAGCTCTGATGG  
             | Rev - GTCCAGGTCATTCAACATCAAGTTC |
| rPGC-1β   | For - TTTGGCTCTCTCTCTCAGTGA  
             | Rev - TCCGCCGCTCTCTGG |
| rPPARα    | For - ACTAGGAGTGTCGGAAGCTGAGT  
             | Rev - TTTGCAATGCGGACTGCTGAGC |
| rERRα     | For - TCCGCCGCTGCCAAG  
             | Rev - CACAGCCTCAGCATCTTCAA |
| rMCAD     | For - CCGTCTCTCTCATCAAGAG  
             | Rev - CGTGAAATTAAAACACAGGCAATCAAC |
| rGLUT4    | For - CCCCCGATACCTCCTACAT  
             | Rev - GCATGACACACATGACCGCAG |
| rTNFa     | For - CATCTTTCATCAACAAAATTCGAGTGACAA  
             | Rev - TGGGAGTAGATAGAAGTCGAGC |
| rIL-6     | For - ACCACCCACACAGCAGAGT  
             | Rev - CAGAATTGCGATTGCAACAC |
Supplemental Figure 1

(a) Expression of various genes over time:

- **LCAD**
- **VLCAD**
- **NRF2**
- **COX4**
- **Atp Syn**
- **CD36**
- **GLUT1**
- **PDK4**
- **PPARβ/δ**

Expression is shown in relative units for each time point (0, 6, 12, 24 hours). Bars indicate baseline, PBS, and LPS conditions with asterisks (*) indicating significant differences.

(b) Western blot analysis:

- **AMPK-P**
- **AMPK-total**

Protein expression levels are compared between PBS and LPS conditions.
Supplemental Figure 2

(a) Expression of PGC-1α with treatment of LPS, TNF, and PBS with control (Isotype) and anti-TNFα antibody. 

(b) Expression of PGC-1α with treatment of LPS and PBS with control (Isotype) and anti-IL-6 antibody. 

(c) NO production with treatment of LPS and PBS with vehicle (Veh) and L-NIL.
Supplemental Figure 3

![Expression Levels of TNFα, iNOS, MCP1, and IL6](image)

- **TNFα**: NTG > TG, LPS > PBS
- **iNOS**: NTG > TG, LPS > PBS
- **MCP1**: NTG > TG, LPS > PBS
- **IL6**: NTG > TG, LPS > PBS

* p < 0.05
‡ p < 0.01

Expression (Relative Units)
Supplemental Figure Legends

Supplemental Figure 1. (a) qRT-PCR assessment of myocardial gene expression in C57BL/6 mice at baseline (gray bars) or at various timepoints following injection with LPS (black bars) or PBS (white bars). (b) Total cellular protein from NRCMs treated with PBS or LPS for 18h were analyzed via Western blot with antibodies specific for total or phospho-AMPK. * p <0.05 PBS vs LPS (2-way ANOVA).

Supplemental Figure 2. NRCMs were stimulated with PBS (white bars), LPS (black bars), or TNF-α (gray bars) in presence of a neutralizing antibody against TNF-α (a), IL-6 (b) or control goat IgG and PGC-1α expression was determined via qRT-PCR. To demonstrate the inhibitory effects of the IL-6 antibody, STAT3 phosphorylation was assessed via Western blot using antibodies against total or phospho-STAT3 (b, right panel). (c) NRCMs were pretreated with the iNOS inhibitor LNIL and PGC-1α expression or NO production was determined by qRT-PCR or colorimetric assay, respectively. * p <0.05 compared to control (2-way ANOVA).

Supplemental Figure 3. Inflammatory cytokine production after LPS stimulation is not reduced in TRE-PGC1βMHCrtTA mice. TRE-PGC-1βMHCrtTA transgenic (TG) mice or non-transgenic littermates (NTG) were fed with doxycycline chow for 48h and then injected with PBS (white bars) or LPS (black bars). Myocardial mRNA was isolated at 2h (TNFα, MCP1, IL-6) or 6h (iNOS) after LPS injection and gene expression was assessed by qRT-PCR. All results are normalized to 36B4; n= 5-8 per group, bars represent mean ± SE; * p <0.05 PBS vs. LPS; † p <0.05 NTG vs. TG (2-way ANOVA).
Supplemental References


