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Original Contribution

Nitric oxide synthase-2 induction optimizes cardiac mitochondrial biogenesis after endotoxemia

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ABSTRACT

Mitochondrial biogenesis protects metabolism from mitochondrial dysfunction produced by activation of innate immunity by lipopolysaccharide (LPS) or other bacterial products. Here we tested the hypothesis in mouse heart that activation of toll-like receptor-4 (TLR4), which induces early-phase genes that damage mitochondria, also activates mitochondrial biogenesis through induction of nitric oxide synthase (NOS2). We compared three strains of mice: wild type (Wt) C57BL/6J, TLR4$^{-/-}$, and NOS2$^{-/-}$for cardiac mitochondrial damage and mitochondrial biogenesis by real-time RT-PCR, Western analysis, immunochemistry, and isoform analysis of myosin heavy chain (MHC) after sublethal heat-killed *Escherichia coli* (*HkEC*). After *HkEC*, Wt mice displayed significant myocardial mtDNA depletion along with enhanced TLR4 and NOS2 gene and protein expression that normalized in 72 h. *HkEC* generated less cytokine stress in TLR4$^{-/-}$and NOS2$^{-/-}$than Wt mice, NOS2$^{-/-}$mice had mtDNA damage comparable to Wt, and both knockout strains failed to restore mtDNA copy number because of mitochondrial transcriptosome dysfunction. Wt mice also showed the largest β-MHC isoform switch, but MHC recovery lagged in the NOS2$^{-/-}$and TLR4$^{-/-}$strains. The NOS2$^{-/-}$mice also unexpectedly revealed the codependency of TLR4 expression on NOS2. These findings demonstrate the decisive participation of NOS2 induction by TLR4 in optimization of mitochondrial biogenesis and MHC expression after gram-negative challenge.

Introduction

Cardiovascular collapse and microvascular deregulation are two major consequences of severe sepsis that lead to shock and poor tissue perfusion. Even after resuscitation, nearly half of patients with severe sepsis die from multiple organ dysfunction syndrome (MODS), the most common cause of death in the critically ill [1]. Earlier studies have implicated mitochondrial dysfunction in the pathogenesis of organ damage and failure in sepsis by mechanisms that are not yet well understood [2].

The innate immune response provides the initial defense against sepsis; for instance, gram-negative microbes activate Toll-like receptor-4 (TLR4) by recognition of lipopolysaccharide (LPS). LPS binds TLR4 in conjunction with LPS-binding protein and CD14 to activate nuclear factor-κB (NF-κB) and major proinflammatory pathways [3–6]. The early phase, mediated by Toll-IR-1R (TIR) and the MyD88 adaptor protein, promotes gene expression for inflammatory cytokines such as TNF-α and IL-1β, as well as the inducible nitric oxide synthase (NOS2) [6,7]. These events generate reactive oxygen and nitrogen species (ROS, RNS) which inhibit respiration, damage mitochondrial DNA (mtDNA), and decrease mtDNA copy number [8–10]. The early phase of sepsis is also associated with cardiac depression, thus implicating TLR4 in the cardiovascular dysfunction [11,12]. Affirmatively, TLR4-deficient mice are protected from mitochondrial dysfunction, cardiomyocyte impairment, [13] and LPS-induced shock [14].

To counteract microbial challenges, multiple host genes are induced that are needed to maintain oxidative phosphorylation, mitochondrial DNA transcription and replication, and mitochondrial biogenesis. Under these conditions, compensatory mitochondrial biogenesis is driven in part by TLR4-dependent ROS production, consistent with the antipodal effects of oxidative stress on mitochondria [15]. Thus, mitochondrial ROS production is not only a cause of oxidative damage but also a signal to activate mitochondrial biogenesis. The molecular program is regulated at the transcriptional level by the nuclear respiratory factors (NRF-1 and NRF-2), the PGC-1 family of coactivators, and the mitochondrial transcription factor,

Abbreviations: cyt *b*, cytochrome *b*; GFP, green fluorescent protein; *HkEC*, sublethal heat-killed *Escherichia coli*; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; MHC, myosin heavy chain; NO, nitric oxide; NOS, nitric oxide synthase; NRF, nuclear respiratory factors; RNS, reactive nitrogen species; ROS, reactive oxygen species; TLR4, toll-like receptor-4; Wt, wild type.

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Tfam [16,17]. Its failure is presumed to lead to cell dysfunction and delayed organ recovery due to mtDNA damage and disturbances in oxidative phosphorylation [18,19].

The main source of NO in sepsis is NOS2, and NO overproduction is known to depress mitochondrial and cardiac function, may impair cardiomyocyte survival, and has been linked to heart failure and poor clinical outcomes [20–22]. However, NOS2 also confers protective antiapoptotic effects [23]; for instance, NOS2 induction by TLR4 is required for functional rescue and survival of cardiomyocytes after LPS [24]. In other settings, NOS3 and NOS1 protect the cell by initiating mitochondrial biogenesis by activation of guanylate cyclase and cGMP-dependent PGC-1α expression [25–29]. However, no comparable role for NOS2 in cardiac protection has been identified.

Because LPS stimulates NO production via TLR4 activation, we tested the hypothesis that TLR4 and NOS2 interact to provide cardiac mitochondrial protection in mice challenged with *E. coli*. We expected that TLR4-dependent NOS2 up-regulation would improve the expression of critical proteins required for transcriptional regulation of mitochondrial biogenesis, including NRF-1, PGC-1α, and Tfam. Furthermore, we hypothesized that the mitochondrial response to TLR4 and NOS2 stimulation would be integrated functionally with isoform switching in the myosin heavy chain (MHC). To investigate these ideas, we evaluated cardiac mitochondrial damage and quantified mRNA and proteins involved in regulating mitochondrial biogenesis in wild-type and TLR4-and NOS2-deficient mice before and after acute systemic administration of heat-killed *Escherichia coli (HkEC)*.

Materials and methods

Animal studies

TLR4$^{-/-}$mice were generously provided by Dr. S. Akira of Osaka University [30,31] and then speed congenically backcrossed onto a C57BL/6J background. NOS2$^{-/-}$and Wt C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and the NOS2 mice bred at our institution. For immunofluorescence studies, we used transgenic reporter mice that express green fluorescent protein (GFP) exclusively in mitochondria (mtGFP-tg), a gift from Hiroshi Shitara and Hiromichi Yonekawa of Tokyo Metropolitan Institute of Medical Science [32]. Mice were kept in pathogen-free housing on dry bedding and studies were conducted with 20–25 g males on approved protocols that conform to the NIH *Guide for the Care and Use of Laboratory Animals*.

Live bacteria were prepared from lyophilized *E. coli* (serotype 086a: K61, American Type Tissue Culture Collection, Rockville, MD) as described [33], carefully heat-inactivated to avoid disruption, and stored at−80 °C until use. These inactivated bacteria (*HkEC*) were thawed once and diluted with sterile 0.9% NaCl to a concentration of 1×10^7/ml and 0.5 ml was administered by single intraperitoneal injection to groups of 3 to 6 mice per time point. Preliminary studies showed that this sublethal dose of bacteria produces minimal depression of peak respiration in isolated cardiac mitochondria and is below the threshold for cardiomyocyte necrosis in these mouse strains.

Cardiac harvest

Mice injected with *HkEC* were killed under anesthesia at 0, 6, 24, 48, and 72 h and the hearts collected immediately. The hearts were flash-frozen in liquid nitrogen for all mRNA and protein analysis and stored at −80 °C until processed. Hearts for immunofluorescence studies were fresh-fixed in 10% formalin for 24 h and then stored in 70% ethanol and phosphate-buffered saline until processed as described below.

Real-time PCR and gene expression

Total cardiac RNA was isolated from tissue using Trizol Reagent (Invitrogen, Carlsbad, CA) and cDNA synthesized using the SUPERSCRIPT Choice System Kit (Invitrogen). Mouse-specific primers and probes (Table 1) were designed using Primer Express (Applied Biosystems, Branchburg, NJ). Reactions to quantify per sample levels of TNF-α, IL-6, IL-1β, ICAM-1, NOS2, ND1, and COX1 were carried out on a 7700 Sequence Detector System (Applied Biosystems) as described [10]. β-MHC and TLR-4 mRNA expression was determined as described [34] using gene-specific primer pairs (Table 1). Each sample was assayed in triplicate and mean values were reported. The RNA data were normalized to 18S rRNA expression levels.

Mitochondrial isolation, respiration, and DNA copy number

Intact mitochondria were isolated from fresh hearts using sucrose density centrifugation [16]. Respiration was measured at 35 °C in water-jacketed cuvettes with calibrated polarographic oxygen electrodes (Diamond General, Ann Arbor, MI) using 0.5 mM ADP and either succinate (5 mM) or malate+ glutamate (2.5 mM each) as substrates. MtDNA was isolated from intact mitochondria [15,34] and the copy number quantified using real-time PCR for cytochrome *b* (cyt *b*) as described [10]. Samples were analyzed in triplicate and the mtDNA copy number per nanogram total cellular DNA was reported by logarithmic expression relative to known DNA standards.

Western blot analysis

To prepare cardiac tissue for Western blotting, hearts were divided longitudinally so that atrial and ventricular tissues were represented in each sample. Proteins were separated by SDS-PAGE and transferred to PVDF membranes for Western analysis [16,34]. Membranes were incubated with validated polyclonal or monoclonal antibodies against PGC-1α, NRF1, Tfam, Pol-γ, CD68, Akt, pAkt, AMPK, and p-AMPK (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-tubulin or β-actin (1:1000; Sigma) was used as a loading control. After three washes in TBST, membranes were incubated in HRP-conjugated goat anti-rabbit or anti-mouse IgG (Santa Cruz) at 1:2000 or 1:5000 dilutions, respectively. Blots were developed with ECL and proteins quantified by densitometry of digitized images from the mid-dynamic range and expressed relative to β-actin or tubulin.

Table 1
Gene primers and probe sequences

TNF-α	FW: ACGGCATGGATCTCAAAGAC
	RV: GTGGGTGAGGAGCACGTAGT
	Probe: FAM-5′-CTCTTCAAGGGACAAGGCTG-3′-MGB
IL-6	FW: CCGGAGAGGAGACTTCACAG
	RV: TCCACGATTTCCCAGAGAAC
	Probe: FAM-5′-ACCACTTCACAAGTCGGAGG-3′-MGB
Cyt *b*	Cyt *b*-s: ACCCTAGTCGAATGAAT
	Cyt *b*-as: TCTGAGTTTAATCCTGT
WT mtDNA	Mt1F: AGGGATCCCACTGCACATAG
	Mt2R: CTCCTCATGCCCCTATGAAA
	Probe: FAM-5-TTTAATTCAAATTTACCCGCTACTCAACTCTACTATCATTT
	TAA-3′-MGB
Deleted	Dl1F: TGGCCTACACCCAGAAGATT
mtDNA	Dl2R: TGTTTTCTTAGGGCTTTGAAGG
	Probe: VIC-5′-TCATGACCAATGAACACTCTGACCCAACTAATTAC-3′-TAMRA
TLR-4	FW: ACCTGGCTGGTTTACACGTC
	RV: CTGCCAGAGACATTGCAGAA
NOS2	FW: CACCTTGGAGTTCACCCAGT
	RV: ACCACTCGTACTTGGGATGC
ND1	FW: CACCCCCTTATCAACCTCAA
	RV: ATTTGTTTCTGCGAGGGTTG
COX I	FW: GGAGCAGTATTCGCCATCAT
	RV: GAGCACTTCTCGTTTTGATGC
18S rRNA	FW: Tcaatctcgggtggctgaacg
	RV: GGACCAGAGCGAAAGCATTTG