MyD88 and NOS2 are essential for Toll-like receptor 4-mediated survival effect in cardiomyocytes

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Zhu, Xinsheng, Huailong Zhao, Amanda R. Graveline, Emmanuel S. Buys, Ulrich Schmidt, Kenneth D. Bloch, Anthony Rosenzweig, and Wei Chao. MyD88 and NOS2 are essential for Toll-like receptor 4-mediated survival effect in cardiomyocytes. Am J Physiol Heart Circ Physiol 291: H1900–H1909, 2006. First published April 28, 2006; doi:10.1152/ajpheart.00112.2006.—Innate immune system such as Toll-like receptor 4 (TLR4) represents the first line of defense against infection. In addition to its pivotal role in host immunity, recent studies have suggested that TLR4 may play a broader role in mediating tissue inflammation and cell survival in response to noninfectious injury. We and other investigators have reported that cardiomyocytes signaling is dynamically modulated in ischemic myocardium and that activation of TLR4 confers a survival benefit in the heart and in isolated cardiomyocytes. However, the signaling pathways leading to these effects are not completely understood. Here, we investigate the role of MyD88, an adaptor protein of TLR4 signaling, and inducible nitric oxide synthase (NOS2) in mediating TLR4-induced cardiomyocyte survival in an in vitro model of apoptosis. Serum deprivation induced a significant increase in the number of apoptotic cardiomyocytes as demonstrated by transferase-mediated dUTP nick-end labeling (TUNEL) assay, nuclear morphology, DNA ladderling, and DNA-histone ELISA. Lipopolysaccharide (LPS), a TLR4 agonist, activated TLR4 signaling and led to significant reduction in apoptotic cardiomyocytes and improved cellular function of surviving cardiomyocytes with enhanced Ca2+ transients and cell shortening. We found that both TLR4 and MyD88 are required for the LPS-induced beneficial effects as demonstrated by improved survival and function in wild-type but not in TLR4−/− or MyD88−/− cardiomyocytes. Moreover, genetic deletion or pharmacological inhibition of NOS2 abolished survival and functional rescue of cardiomyocytes treated with LPS. Taken together, these data suggest that TLR4 protects cardiomyocytes from stress-induced injury through MyD88- and NOS2-dependent mechanisms.

signal transduction; cardiac; apoptosis; inducible nitric oxide synthase

ISCHEMIC HEART DISEASE represents a major cause of morbidity and mortality in Western society. Because injured cardiac tissues after a heart attack typically cannot regenerate, ischemic heart disease often leads to deterioration of myocardial function and death. Therefore, it is critically important and clinically relevant to understand the molecular mechanisms governing ischemic myocardial injury and identify potential targets for intervention.

Cardiomyocyte apoptosis, or programmed cell death, is an important feature of ischemic myocardial injury. With the employment of pharmacological inhibition of apoptosis, transgenic cardiac expression of caspase, and genetic deletion of anti-apoptotic signaling pathway, previous studies have demonstrated that cardiomyocyte apoptosis plays an important role in ischemic myocardial injury and cardiomyopathy (19, 54, 56).

Innate immune signaling via Toll-like receptor 4 (TLR4) and the downstream adapter molecules MyD88 (38) and the serine-threonine kinase, interleukin-1 receptor-associated kinase (IRAK-1) (21) plays an important role in host defense and tissue inflammation (47). The heart expresses at least three receptors involved in TLR signaling: CD14, TLR2, and TLR4 (12, 13, 30, 40). It is well documented that these receptors are responsible for cardiac contractile dysfunction in various pathological conditions such as endotoxemia (30, 40). In contrast, little is known about the role of innate immune signaling in myocardial injury. A few recent studies have found that the cardiac innate immune system is dynamically regulated in response to ischemic injury and may play a role in cardiomyocyte survival. For example, myocardial TLR4 expression is upregulated in response to myocardial ischemia and during heart failure (13). Chao et al. (6) recently found that myocardial ischemia induces rapid activation of IRAK-1, a critical component of TLR innate immune signaling (21) and that adenovirus-mediated expression of IRAK-1 inhibited cardiomyocyte apoptosis in vitro. Moreover, administration of lipopolysaccharide (LPS), a TLR4 agonist, protects the heart against ischemic injury (3, 39) and inhibits cardiomyocyte apoptosis in vitro (6).

MyD88, originally isolated as one of the 12 myeloid differentiation primary response genes (34), is an adaptor protein that plays an important role in TLR-interleukin-1 receptor family signaling. Like TLR4-deficient mice (44), MyD88−/− mice (25) lack the ability to respond to LPS, although MyD88-independent pathways (e.g., TRIF-dependent IFN-β production) exist in TLR4 signaling (26). The role of MyD88 in cell survival or death is not completely understood, and earlier results have been conflicting (1, 23).

Three nitric oxide synthase (NOS) isoforms exist in the heart: neuronal (NOS1), inducible (NOS2), and endothelial (NOS3). With the utilization of genetically modified animal models and pharmacological inhibitors, it has been demonstrated that all three NOs play important roles in modulating cardiac function and survival under various conditions (22, 50,
Myocardial expression of NOS2 is induced in response to endotoxin-cytokine stimulation and to myocardial ischemia (15). NOS2 may contribute to myocardial dysfunction during endotoxemia (50), but its role in cardiac protection against ischemic injury is controversial (11, 15, 32, 46).

In this study, we sought to determine the role of MyD88 and NOS2 in modulating cardiomyocyte survival and function in an in vitro model of apoptosis. Utilizing genetically modified mice, we found that TLR4 mediates a cardiomyocyte survival signal and that both MyD88 and NOS2 are critical components of this signaling mechanism.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were performed with the approval of the Animal Care Committee of Massachusetts General Hospital. MyD88−/− mice (kindly provided by Dr. Mason Freeman, Massachusetts General Hospital) were backcrossed six generations into the C57BL/6J strain (25). TLR4−/− (C57BL/10ScCr also referred to as C57BL/10ScNJ, Stock No. 003752, with wild-type Il12rb2 allele), TLR4+/− (C57BL/10ScSn), and NOS2−/− mice were purchased from The Jackson Laboratory. NOS2−/− mice were backcrossed for 10 generations into the C57BL/6J strain. Unless stated otherwise, C57BL/6J mice were studied as the wild-type controls.

Neonatal Mouse Cardiomyocytes Isolation and Culture

Neonatal mouse cardiomyocytes were prepared from 1- to 3-day-old mice as described previously (52). More than 95% of cells were cardiomyocytes as demonstrated by sarcomeric actin staining 4 days after being plated. Neonatal cardiomyocytes were used for all apoptosis studies.

Adult Mouse Cardiomyocyte Isolation

Ventricular mouse cardiomyocytes were isolated as described previously (33) and used for the function studies: Ca2+ transients and cell shortening.

Measurement of Cardiomyocyte Sarcomere Shortening and Intracellular Calcium

Sarcomere shortening and intracellular calcium concentration transients were recorded simultaneously on an IonOptix system (Milton, MA). Adult cardiomyocytes were incubated with membrane-permeable fluorescent indicator fura-2 AM (1 μM) (Molecular Probes) and probenecid (0.5 mM). Cardiomyocytes were perfused with 1.2 mM Ca2+ Tyrode solution and electrically paced at 2 or 1 Hz via platinum wires. The Ca2+ transients and sarcomere shortening were analyzed based on single cell averaged tracing. The final values were derived from 20–30 individual cells in each group and calculated for statistical analysis. Three to four mice from each group (mouse strain) were employed to prepare cardiomyocytes for the functional studies.

In Vitro Model of Cardiomyocyte Apoptosis

Three to four days after being plated, neonatal cardiomyocytes were washed three times and incubated in serum-free MEM containing 0.05% bovine serum albumin in an incubator containing 5% CO2-95% air at 37°C. We have previously established that serum deprivation induces a rapid activation of caspases (within 4 h) and cardiomyocyte apoptosis (5, 6). In some studies, neonatal cardiomyocytes were exposed to hypoxic condition, which was created using BD Bioscience GasPak EZ system.

Apoptosis Assays

DNA laddering. Neonatal cardiomyocytes (1 × 10⁶) were lysed, and DNA was extracted with phenol:chloroform. Genomic DNA...
fragments of 1.5 μg were 32P-labeled by Klenow DNA polymerase and subsequently separated by electrophoresis in 1.8% agarose gel (6).

Cell death ELISA. Apoptosis-induced histone-DNA fragments were quantitated using ELISA (Roche).

TUNEL and Hoechst nuclear staining. Attached neonatal cardiomyocytes were fixed and permeabilized. Terminal deoxynucleotidyl transferase was used to incorporate fluorescein-labeled dUTP into 3'-OH DNA ends generated by DNA fragmentation (Roche). Nuclei were costained with Hoechst. Actin was stained with FITC-labeled phalloidin (10 μg/ml). To quantitate apoptotic nuclei, 400–900 nuclei in each treatment group from ten ×400 magnification fields were counted. The operator who counted nuclei was blinded to the experimental design.

Immunoblotting, Immunoprecipitation, and IRAK-1 Kinase Assay

These experiments were performed as described in detail previously (6). Briefly, cardiomyocytes were incubated in serum-free medium for 1 h and treated with LPS. IRAK-1 immunoprecipitates were assayed for kinase activity using [γ-32P]ATP and myelin basic protein as the substrates.

Immunocytochemistry

For microscopic image of NOS2, neonatal cardiomyocyte cultures (10^5 cells/chamber on 8-chamber slide) were treated with LPS. Cells were fixed and permeabilized using a cytostaining kit from BD PharMingen (San Diego, CA). NOS2 was stained with a monoclonal antibody (1:100 dilution) (BD Bioscience, Clone 6) at 4°C overnight and with Alexa Fluor 546-labeled anti-mouse IgG (1:2,000) for 1 h at room temperature.

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from cultured cardiomyocytes using TRIzol reagent, and cDNA was synthesized by reverse transcriptase reaction. cDNA gene sequences corresponding to 18S ribosomal RNA (rRNA) were amplified from cDNA by PCR and quantitated using an ABI Prism 7000 (Applied Biosystems) with the forward primer 5'-TCATGTGGTGATTCTGCATAATG-3' and the reverse primer 5'-TGCCGTGGATTCTGATCAATG-3'. NOS2 and NOS3 cDNA sequences were detected using Taqman primer sets supplied by Applied Biosystems. Changes in relative gene expression normalized to 18S rRNA levels were determined using the relative C_T method.
**Characterization of TLR4**<sup>−/−</sup> **and MyD88**<sup>−/−</sup> **Cardiomyocytes**

MyD88 expression was confirmed by PCR (data not shown) and Western blots (Fig. 1A). MyD88 immunoreactivity was detected in the lung, heart, and spleen of wild-type but not MyD88<sup>−/−</sup> mice. MyD88 protein levels were decreased in MyD88<sup>+/−</sup> mice. LPS induced IRAK-1 activation in wild-type cardiomyocytes but not in MyD88<sup>−/−</sup> or TLR4<sup>−/−</sup> cardiomyocytes (Fig. 1B). However, cardiomyocytes isolated from adult wild-type, TLR4<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice shared similar morphology and striation patterns and had similar sarcomere length (Fig. 1, C and D).

**TLR4 and MyD88 are Essential for LPS-Induced Anti-Apoptotic Effect in Cardiomyocytes**

Serum deprivation, an in vitro model of apoptosis, induced significant apoptosis as demonstrated by transferase-mediated dUTP nick-end labeling (TUNEL) staining, nuclear morphology (condensation and fragmentation) (Fig. 2A), ELISA for histone-DNA fragments (Fig. 3), and DNA laddering (Fig. 3). As shown in Fig. 2A, apoptotic cardiomyocytes were characterized by cell shrinking (actin staining), nuclear fragmentation and condensation (Hoechst staining), and positive TUNEL. The serum deprivation-induced apoptosis was time dependent with 1.5 ± 0.9% at 0 min and up to 11.4 ± 3.5% at 48 h. LPS at the concentrations ranging from 10 to 2,500 ng/ml, when added at the time of serum deprivation, led to a significant reduction in the number of apoptotic cells (data not shown). LPS failed to exhibit anti-apoptotic effect when added 3–6 h after serum deprivation started as demonstrated by DNA laddering (data not shown). LPS exhibited similar anti-apoptotic effect in neonatal murine cardiomyocytes exposed to transient hypoxia (Fig. 4). To elucidate the role of TLR4 and MyD88 in mediating the survival effect, we studied TLR4<sup>−/−</sup> and MyD88<sup>−/−</sup> cardiomyocytes. The data indicated that serum deprivation induced a comparable level of cardiomyocyte apoptosis among wild-type, TLR4<sup>−/−</sup>, and MyD88<sup>−/−</sup> cardiomyocytes (Figs. 2 and 3). The apoptotic rates were 9.7 ± 2.7% (n = 3), 9.6 ± 1.3% (n = 5), and 8.6 ± 3.2% (n = 4), respectively, for wild-type, TLR4<sup>−/−</sup>, and MyD88<sup>−/−</sup> cardiomyocytes. The anti-apoptotic effects of LPS (1,000 ng/ml) observed in wild-type cardiomyocytes, however, were abolished in TLR4<sup>−/−</sup> and MyD88<sup>−/−</sup> cardiomyocytes as demonstrated by apoptotic nuclei counting (Fig. 2, C and D), DNA fragmentation ELISA.

**Fig. 3.** Role of TLR4 and MyD88 in LPS-induced survival effect: cell death ELISA and DNA laddering. A: cell death ELISA. Cells were incubated in serum-free MEM containing 0.05% BSA for 8 h. Results were normalized against the control cells incubated in the presence of serum. n = 4, each performed in triplicates. **P < 0.01 vs. cells in the presence of serum; **P < 0.05 vs. cells exposed to SD but without LPS, 1,000 ng/ml; #P < 0.05 vs. cells in the presence of serum. B–D: DNA laddering. LPS (1,000 ng/ml) inhibits SD-induced DNA fragmentation in WT but not in MyD88<sup>−/−</sup> or TLR4<sup>−/−</sup> cardiomyocytes. Similar results were obtained with C57BL/6J WT cardiomyocytes. C: C57BL/10ScSn (TLR4<sup>−/−</sup>) cells (data not shown). D: TLR4<sup>−/−</sup> or MyD88<sup>−/−</sup> cardiomyocytes. LPS (1,000 ng/ml) or IGF-1 (100 nM) was added at the time of SD.
LPS Improves Ca\textsuperscript{2+} Transients and Cell Shortening After Serum Deprivation: Role of TLR4 and MyD88

To assess the function of those surviving cardiomyocytes after serum deprivation and the impact of LPS treatment, we measured Ca\textsuperscript{2+} handling and cell shortening of isolated adult mouse cardiomyocytes. Cardiomyocyte function was significantly inhibited after 3 h of serum deprivation with \~26% reduction in maximal velocity (V\textsubscript{dep}) and 23% in amplitude (ΔF/F0) of Ca\textsuperscript{2+} transients compared with cells incubated in serum-containing medium (data not shown). LPS, added at the time of serum deprivation, significantly improved the function with a 27% increase in both V\textsubscript{dep} and ΔF/F0 and a 21% increase in maximal returning velocity to baseline (V\textsubscript{ret}) (Fig. 5 and Table 1, group II vs. group I). LPS also led to a 50% increase in the V\textsubscript{dep} of sarcomere shortening, a 58% increase in sarcomere shortening, and a 64% increase in V\textsubscript{ret} (Fig. 5 and Table 2, group II vs. group I). It is noteworthy that LPS also improved calcium handling and contractile function of cardiomyocytes in the presence of serum (data not shown). To test whether TLR4 and MyD88 mediate the function improvement induced by LPS in the serum deprivation model, we studied adult TLR4\textsuperscript{-/-} and MyD88\textsuperscript{-/-} cardiomyocytes. As shown in Fig. 5 and Tables 1 and 2, compared with wild-type cardiomyocytes, TLR4\textsuperscript{-/-} and MyD88\textsuperscript{-/-} cardiomyocytes showed similar response to electric pacing. Importantly, unlike wild-type cardiomyocytes, neither TLR4\textsuperscript{-/-} nor MyD88\textsuperscript{-/-} cardiomyocytes responded to LPS treatment with no detectable difference in all Ca\textsuperscript{2+} transient and cell shortening parameters between LPS-treated and the control cells (Tables 1 and 2, group IV vs. group III and group VI vs. group V).

NOS2 is Critical for TLR4-Mediated Survival and Function Recovery After Serum Deprivation

To examine the role of NOS2 in the TLR4-induced beneficial effects by LPS, we utilized the selective NOS2 inhibitor N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) and cardiomyocytes from mice congenitally deficient for NOS2 (31). We first tested the TLR4 dependency of the cardiac NOS2 induction in response to LPS administrated intraperitoneally. Eight hours after systemic administration of LPS, there was an increase in NOS2 protein in the heart from wild-type mice but not from that of TLR4\textsuperscript{-/-} or NOS\textsuperscript{-/-} mice (Fig. 6a,b). Moreover, to demonstrate the specific phenotypic effects of the gene knockout in these mice, we also tested the IRAK-1 kinase activity in response to LPS. As anticipated, the IRAK-1 activation is severely impaired in TLR4-deficient mice but remained largely intact in NOS2\textsuperscript{-/-} mice (Fig. 6a,e), suggesting that the upstream signaling of NOS2\textsuperscript{-/-} mice is intact. Similarly, in cultured wild-type neonatal cardiomyocytes, LPS induced a rapid induction of NOS2 mRNA, but not NOS3, in the heart from wild-type mice but not from that of TLR4\textsuperscript{-/-} or NOS\textsuperscript{-/-} mice (Fig. 7A). The NOS2 protein expression was also increased in neonatal cardiomyocytes in response to LPS as demonstrated by immunocytochemistry and immunoblotting (Fig. 6, C and D).

As shown in Fig. 7A, serum deprivation for 24 h led to a fourfold increase in apoptotic cells (2.3 ± 0.6% of the control vs. 9.6 ± 1.7% of serum deprivation). LPS inhibited the serum deprivation-induced apoptosis in control cells (9.6 ± 1.7% in serum deprivation cells vs. 4.0 ± 0.8% in serum deprivation cells treated with LPS, P < 0.01). However, in the presence of L-NIL, the LPS-induced survival benefit was blocked (7.8 ± 1.8% of cardiomyocytes exposed to serum deprivation + L-NIL vs. 5.7 ± 1.7% of cells exposed to LPS + serum deprivation + L-NIL, P > 0.1, n = 4). L-NIL had no significant effect on the level of apoptotic cells in serum or on the level of apoptosis induced by serum deprivation. IGF-1-induced survival effect was not affected by L-NIL (Fig. 7A). Moreover, TLR4 activation failed to inhibit serum deprivation-induced apoptosis in the NOS2\textsuperscript{-/-} cardiomyocytes as demonstrated by DNA laddering (Fig. 7B), by apoptotic nuclei counting (Fig. 7C), and by histone-DNA fragment ELISA (Fig. 7D). In
In contrast, IGF-1 maintained its anti-apoptotic effect in the NOS2−/− cardiomyocytes (Fig. 7, C and D). We next examined whether NOS2 also plays a role in TLR4-mediated functional improvement observed in wild-type cardiomyocytes. As shown in Fig. 5 and Tables 1 and 2 (group VIII vs. group VII), LPS failed to improve Ca2+ transients and cell shortening under serum-free conditions in NOS2−/− cardiomyocytes.

**DISCUSSION**

Previous studies have found that LPS, a TLR4 agonist, confers a cardioprotective effect in both isolated hearts and cultured cardiomyocytes (3, 6, 39). However, the signaling pathways leading to the cardiac protection are not completely understood. In the present study, we explored the role of TLR4

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**Table 1. Analyzed Ca2+ transient parameters in WT, TLR4−/−, MyD88−/−, and NOS2−/− cardiomyocytes**

<table>
<thead>
<tr>
<th>Serum</th>
<th>LPS</th>
<th>V_{dep}, U/s</th>
<th>ΔF/F₀, %</th>
<th>V_{ret}, U/s</th>
<th>tau, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>–</td>
<td>26.8±12.1</td>
<td>21.9±7.7</td>
<td>−3.3±1.0</td>
<td>0.098±0.043</td>
</tr>
<tr>
<td>Group II</td>
<td>– +</td>
<td>34.2±12.2*</td>
<td>27.9±7.7*</td>
<td>−4.0±1.6*</td>
<td>0.076±0.013</td>
</tr>
<tr>
<td>TLR4−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>–</td>
<td>57.2±27.1</td>
<td>31.4±12.1</td>
<td>−7.2±7.4</td>
<td>0.111±0.054</td>
</tr>
<tr>
<td>Group IV</td>
<td>– +</td>
<td>46.5±15.7</td>
<td>25.6±12.2</td>
<td>−5.9±3.4</td>
<td>0.119±0.074</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group V</td>
<td>–</td>
<td>26.1±14.4</td>
<td>22.5±10.4</td>
<td>−4.4±3.4</td>
<td>0.095±0.025</td>
</tr>
<tr>
<td>Group VI</td>
<td>– +</td>
<td>30.8±15.7</td>
<td>24.9±10.9</td>
<td>−3.9±3.5</td>
<td>0.108±0.038</td>
</tr>
<tr>
<td>NOS−/−</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Group VII</td>
<td>–</td>
<td>32.3±16.7</td>
<td>28.9±13.1</td>
<td>−3.1±1.8</td>
<td>0.132±0.039</td>
</tr>
<tr>
<td>Group VIII</td>
<td>– +</td>
<td>34.9±18.4</td>
<td>29.6±13.5</td>
<td>−2.9±2.0</td>
<td>0.134±0.044</td>
</tr>
</tbody>
</table>

Values are means ± SD. LPS, lipopolysaccharide; WT, wild-type; TLR4, Toll-like receptor-4; NOS, nitric oxide synthase; V_{dep}, maximal velocity departing from baseline; ΔF/F₀, amplitude change (%) of Ca2+ transients; V_{ret}, maximal velocity returning to baseline; tau, time constant, fit single exponential to baseline. Data in each group were recorded from 20 to 30 single adult cardiomyocytes and analyzed. At least three mice were used in each group to prepare isolated cardiomyocytes. *P < 0.05 compared with control without LPS treatment within each pair of groups, e.g., group II vs. group I or group IV vs. group III.
and assayed for IRAK-1 kinase activity (Fig. 6. LPS induces NOS2 induction.

The heart expresses at least three pattern recognition receptors for a group of microorganisms with pathogen-associated signal and preserves cardiomyocyte function through both MyD88- and NOS2-dependent mechanisms.

and its downstream molecules MyD88 and NOS2 in mediating cardiomyocyte survival and function in an in vitro model of apoptosis. Using genetically modified mice, we demonstrate that cardiomyocyte TLR4 mediates a direct anti-apoptotic

Table 2. Analyzed sarcomere shortening parameters in WT, TLR4−/−, MyD88−/−, and NOS2−/− cardiomyocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum</th>
<th>LPS</th>
<th>Sarcomere Length, μm</th>
<th>$V_{dep}$, μm/s</th>
<th>Sarcomere Shortening, %</th>
<th>$V_{con}$, μm/s</th>
<th>$\tau_s$, s</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>−</td>
<td>−</td>
<td>1.82 ± 0.04</td>
<td>−0.96 ± 0.44</td>
<td>1.30 ± 0.68</td>
<td>0.50 ± 0.31</td>
<td>0.098 ± 0.043</td>
</tr>
<tr>
<td>Group I</td>
<td>−</td>
<td>+</td>
<td>1.82 ± 0.07</td>
<td>−1.44 ± 0.66*</td>
<td>2.06 ± 0.86*</td>
<td>0.82 ± 0.49*</td>
<td>0.076 ± 0.013</td>
</tr>
<tr>
<td>TLR4−/−</td>
<td>−</td>
<td>−</td>
<td>1.81 ± 0.04</td>
<td>−2.88 ± 1.48</td>
<td>3.82 ± 1.78</td>
<td>1.90 ± 1.43</td>
<td>0.047 ± 0.026</td>
</tr>
<tr>
<td>Group II</td>
<td>−</td>
<td>+</td>
<td>1.79 ± 0.05</td>
<td>−2.54 ± 1.40</td>
<td>3.60 ± 1.97</td>
<td>1.66 ± 1.13</td>
<td>0.051 ± 0.032</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>−</td>
<td>−</td>
<td>1.81 ± 0.05</td>
<td>−2.53 ± 1.20</td>
<td>3.71 ± 1.53</td>
<td>1.68 ± 1.07</td>
<td>0.134 ± 0.145</td>
</tr>
<tr>
<td>Group V</td>
<td>−</td>
<td>+</td>
<td>1.81 ± 0.06</td>
<td>−2.44 ± 1.48</td>
<td>3.33 ± 1.70</td>
<td>1.42 ± 0.96</td>
<td>0.117 ± 0.119</td>
</tr>
<tr>
<td>NOS2−/−</td>
<td>−</td>
<td>−</td>
<td>1.78 ± 0.08</td>
<td>−2.01 ± 1.40</td>
<td>3.50 ± 1.95</td>
<td>1.34 ± 1.27</td>
<td>0.087 ± 0.073</td>
</tr>
<tr>
<td>Group VII</td>
<td>−</td>
<td>+</td>
<td>1.77 ± 0.05</td>
<td>−2.09 ± 1.24</td>
<td>3.98 ± 2.21</td>
<td>1.21 ± 0.93</td>
<td>0.069 ± 0.035</td>
</tr>
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</table>

Values are means ± SD. The data in each group were recorded from 20 to 30 single adult cardiomyocytes and analyzed. At least three mice were used in each group to prepare isolated cardiomyocytes. *P < 0.05 compared with the control without LPS treatment within each pair of groups.

Fig. 6. LPS induces NOS2 induction. A: LPS induces cardiac IRAK-1 activation and NOS2 induction in adult mice. Mice were injected intraperitoneally with LPS (50 mg/kg) (L) or PBS (P). Eight hours later, the hearts were removed and the myocardial tissues analyzed for IRAK-1 kinase activity (a) and for NOS2 protein expression (b). a: IRAK-1 kinase assay. Myocardial tissues were immunoprecipitated for IRAK-1. The IRAK-1 immunoprecipitates were then divided and assayed for IRAK-1 kinase activity (top) or Western blot (bottom). b: NOS2 immunoblotting. Myocardial proteins (150 μg) were loaded in each lane, separated in 4–20% gradient SDS-PAGE, and blotted for NOS2. Actin was blotted to ascertain equal loading of cardiomyocyte samples. **P < 0.05. C: immunocytochemistry of NOS2 in cultured mouse neonatal cardiomyocytes. Cardiomyocytes were incubated without (a) or with (b) LPS (1,000 ng/ml) for 24 h. Cells were then fixed, permeabilized, and immunostained with monoclonal anti-NOS2 antibody (1:100). Nuclei were costained with Hoechst shown in blue. D: NOS2 protein expression in cultured neonatal cardiomyocytes. Cells were incubated without (lane 1) or with (lane 2) LPS (500 ng/ml) for 24 h. Cells were lysed and NOS2 protein immunoblotted. Cell lysates of LPS/IFNγ-treated macrophages (MØ) (BD Bioscience) were used as the positive control for NOS2 with a molecular mass of 130 kDa (lane 3). Actin was blotted to ascertain equal loading of cardiomyocyte samples (lanes 1 and 2).
molecular patterns, including CD14, TLR4, and TLR2 (12, 13, 30, 40). The role of pattern recognition receptors in cell death or survival is not completely understood. Frantz et al. (12) reported that blocking of TLR2 signaling by using a specific antibody increases hydrogen peroxide-induced cell death in vitro, suggesting an anti-apoptotic role for TLR2 in cardiomyocytes (12). In a noninfectious lung injury model, a recent study has demonstrated a proinflammatory and prosurvival role for both TLR2 and TLR4 (23). The study found that extracellular matrix hyaluronan, produced in response to lung injury, acts on both TLR2 and TLR4 to stimulate inflammatory cytokine production and to confer an anti-apoptotic effect in lung epithelial cells in vivo (23). Consistent with this report, our study in TLR4-deficient cardiomyocytes demonstrates that TLR4 mediates a direct survival benefit against serum deprivation-induced apoptosis. Different from the current study, a previous report found that TLR4-deficient mice exhibit reduced tissue inflammation and myocardial infarction after ischemia-reperfusion, suggesting a role of TLR4 in mediating myocardial tissue inflammation and injury (43). The reason for the difference is unclear. We hypothesize that the systemic deficiency of TLR4 could have confounded the in vivo model. The lack of TLR4 in extracardiac sources such as macrophages, lymphocytes, and neutrophils could have direct impact on the myocardial inflammation and injury after ischemia-reperfusion. Recent studies have supported the notion that the extracardiac source of TLR4 plays an important role in mediating cardiac injury and dysfunction under certain pathological conditions such as endotoxemia (49). Therefore, direct contribution of cardiac TLR4 activation to myocardial inflammation and injury after transient ischemia in vivo remains to be determined.

The present study also provides evidence that LPS elicits a direct function preserving, rather than depressing, effect in cardiomyocytes and that TLR4, MyD88, and NOS2 play a critical role in mediating the functional rescue of surviving cardiomyocytes after serum deprivation. It is unclear whether the functional recovery is the result of improved cell survival or vice versa. It is possible that the function-preserving benefit of TLR4 activation may not be the results of improved cell survival but rather contribute to cardiomyocyte survival. It has been reported that improved calcium handling (e.g., by overexpressing sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase pump) decreases ventricular arrhythmias and may contribute to reduced myocardial infarction after ischemia-reperfusion (7).

To dissect the TLR4-mediated signaling pathways that are responsible for LPS-induced survival and function improvement in isolated cardiomyocytes, we utilized mice genetically deficient for MyD88 (25). These mice lack the ability to respond to LPS as measured by B cell proliferation and cytokine production. However, MyD88-independent pathways exist in TLR4 signaling (26). We found that TLR4 activation failed to protect the MyD88\textsuperscript{-/-} cardiomyocytes against serum deprivation-induced apoptosis. To determine whether or not other survival pathways are intact in MyD88\textsuperscript{-/-} cells, IGF-1 was used as a control to block apoptosis and protects cardiomyocytes (4). The survival benefit of IGF-1 is dependent on activation of phosphatidylinositol 3\textsuperscript{-kinase and Akt (PKB)} (10, 55). These data suggest that genetic deletion of MyD88
selectively abolishes the TLR4-mediated cardiomyocyte survival without affecting IGF-1 anti-apoptotic signaling. Our finding that endogenous MyD88 transmits a survival signal in cardiomyocytes is consistent with an in vivo study that demonstrates an anti-apoptotic effect of MyD88 during acute lung injury (23). The study shows that genetic deletion of MyD88, as used in the present study, leads to an increase in the number of apoptotic lung epithelial cells subjected to bleomycin (23). Furthermore, in support of the role of MyD88 as an anti-apoptotic transducer is our recent finding that adenovirus-mediated overexpression of IRAK-1, a kinase recruited by MyD88 during TLR4 activation, confers an anti-apoptotic effect in isolated rat cardiomyocytes (6).

Nitric oxide has been implicated as both a pro- and anti-apoptotic mediator. The pro-apoptotic role of nitric oxide has been largely based on the studies utilizing high levels of exogenous nitric oxide donors and nonspecific NOS inhibitors and therefore its physiological significance is uncertain (2, 20). On the other hand, studies using NOS2−/− mice have yielded seemingly conflicting results with regard to the role of endogenous NOS2 in cardioprotection against ischemia and reperfusion (11, 15, 46). In an infarct model (no reperfusion), NOS2 deletion did not impact infarct size or the level of apoptosis (46). However, using the same genetically modified mouse, Guo et al. (15) demonstrated that NOS2 is required for ischemic preconditioning-induced cardiac protection. With the use of NOS2 gene transfer, the same group (32) has demonstrated that NOS2 overexpression is sufficient to protect myocardium against ischemic and reperfusion injury. In the present study, we sought to examine the contribution of NOS2 to the TLR4-mediated anti-apoptotic effect. Our data suggest that although NOS2 inhibition or genetic deletion has no significant effect on the level of apoptosis induced by serum deprivation, presence of NOS2 is essential for the survival and functional improvement conferred by TLR4 activation. Further study is needed to explore how NOS2 mediates the TLR4-induced cardiomyocyte survival. Both cGMP-dependent and -independent signaling pathways have been suggested to contribute to nitric oxide-mediated cell survival (20, 45, 48). Of particular interest is the recent finding that nitric oxide modulates caspase activity by direct interaction with thiol groups (-SH), a process termed S-nitrosylation (17, 27, 35, 36). However, the role of endogenous NOS and nitric oxide in caspase S-nitrosylation remains to be determined.

Whereas increasing evidence from animal studies indicates a cardioprotective role for NOS2 in myocardial ischemia-reperfusion injury (24), the role NOS2 in human cardiac conditions is much less clear (8). Cardiac NOS2 expression is enhanced in certain cardiac conditions such as ischemic and dilated nonischemic cardiomyopathy (9, 14, 16). However, the significance of NOS2 induction in the pathogenesis of these conditions remains unclear. Increased NOS2 and nitric oxide may attenuate cardiac sensitivity to β-adrenergic stimulation and modulate cardiac contraction (8). On the other hand, increased NOS2, along with NOS3, has been linked to improved left ventricular diastolic function in patients with dilated cardiomyopathy (18).

Some limitations of the present study should be noted. These studies utilized serum deprivation as an in vitro model of apoptosis, which may not reflect the complex nature of myocardial ischemia in the intact animal. Whereas cardiomyocyte cultures offer some advantages over in vivo models, such as avoiding systemic confounders present in the knockout animals, the physiological significance of the present findings needs to be confirmed in animal models.

In summary, in addition to its role in host immunity, recent studies have suggested that TLR4 signaling may play a broader role in mediating tissue inflammation and repair by recognizing various endogenous mediators (23, 41, 51). Some of these endogenous mediators are produced in response to myocardial ischemia (29, 37) and have potent anti-ischemic and anti-apoptotic effects (28, 42). The current studies present clear evidence that activation of TLR4 signaling via MyD88 and NOS2 confers a survival benefit in cardiomyocytes and suggest a potentially important role for TLR4 signaling as an intrinsic cardiac protective mechanism. Identification and characterization of this survival pathway may provide novel targets for intervention for ischemic myocardial injury.

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