Lipopolysaccharide Improves Cardiomyocyte Survival and Function after Serum Deprivation*

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Toll-like receptor-4 (TLR4) and its signaling molecule interleukin-1 receptor-associated kinase (IRAK-1) play an important role in host defense and tissue inflammation. Intriguingly, systemic administration of lipopolysaccharide (LPS), the agonist for TLR4, confers a cardio-protective effect against ischemic injury. However, the mechanisms leading to the cardiac protection remain largely unknown. The present study was designed to investigate the role of TLR4 activation by LPS in protecting cardiomyocytes (CM) against apoptosis in an in vitro model of ischemia and to explore the downstream mechanisms leading to the protective effect. Incubation with LPS led to activation of IRAK-1 and protected CMs against serum deprivation (SD)-induced apoptosis as demonstrated by DNA laddering, histone-DNA fragment enzyme-linked immunosorbent assay, and activation of caspase-3. Phosphatidylinositol 3-kinase/Akt, extracellular signal-regulated kinase 1/2, and IκB kinase β appear to contribute to the anti-apoptotic effect of LPS since the specific inhibitors, wortmannin, PD98059, and dominant negative IKKβ transgene expression reversed the LPS effect. To assess whether LPS improves CM function, we examined intracellular Ca2+ transients and cell shortening in single adult rat CMs. SD for 6 h dramatically inhibited Ca2+ transients and CM contractility. LPS at 500 ng/ml significantly improved the [Ca2+]i transients and enhanced contractility in control CMs as well as in CMs subjected to SD. Importantly, transient ischemia led to rapid activation of IRAK-1 in cultured CMs and in adult rat myocardium. Adenovirus-mediated transgene expression of IRAK-1 but not its kinase-deficient mutant IRAK-1(K239S) protected CMs against SD-induced apoptosis. Taken together, these data suggest an important role of TLR4 signaling via IRAK-1 in protecting against SD-induced apoptosis.

Studies employing pharmacologic inhibitors, transgene expression, and genetically modified animals have demonstrated that cardiomyocyte (CM)1 apoptosis plays an important role in ischemic myocardial injury (1–4). CM apoptosis has been found in injured myocardium in patients who died of myocardial infarction (5–7) and in animal models of infarction (8, 9) and ischemia reperfusion (10).

Apoptosis is a tightly regulated and energy-dependent process that requires specialized cellular machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases (11). The biochemical and cellular hallmarks of apoptosis are characterized by nuclear and DNA fragmentation and condensation, membrane blebbing, and cellular shrinkage.

LPS signal transduction involves multiple signaling proteins such as LPS binding protein, CD14, MD-2, and TLR4. TLR4 dimerizes and becomes activated upon association with LPS-MD2-CD14 complex. Activated TLR4 recruits downstream the serine-threonine kinases, IRAKs, through the adapter protein MyD88. All IRAKs are multidomain proteins consisting of a conserved N-terminal death domain and a central kinase domain (12). The death domain is a protein interaction motif implicated in binding of IRAKs to the upstream adapter protein MyD88. The recruited IRAK-1 becomes multiply phosphorylated, either by IRAK-1 itself or by IRAK-4. Phosphorylated IRAK-1 has reduced affinity for MyD88 and increased affinity for TRAF-6. A point mutation in the ATP binding pocket (K239S) creates a catalytically inactive IRAK-1, IRAK-1(K239S) (13). IRAK-1 and TRAF-6 complex then activate TAK1 through a process involving cytosol translocation of TAK1 and two TAK1-binding proteins from membrane to cytosol and ubiquitination of TRAF-6. Activated TAK1 then phosphorylates IKKα/β as well as mitogen-activated protein kinase (MAPK) kinases, leading to activation of NF-κB and c-Jun NH2-terminal kinase/p38 MAPKs, respectively (14). LPS also activates ERK in macrophages (15) and endothelial cells (16).

Although innate immunity signaling components such as TLR4 are present predominantly in immune cells (17), other tissues and organs such as the heart also possess functionally intact innate immune systems (18, 19). The heart expresses at least three receptors involving TLR signaling: CD14, TLR2, and TLR4 (18–20). A growing body of evidence suggests that the myocardial innate immune system may play an important role in the myocardial innate immune system may play an important role in ischemic myocardial injury (1–4). CM apoptosis has been found in injured myocardium in patients who died of myocardial infarction (5–7) and in animal models of infarction (8, 9) and ischemia reperfusion (10).
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role in mediating the inflammatory response and modulating cardiac function in response to LPS (21, 22). Intriguingly, evidence from several lines of investigation suggests that LPS may activate intracellular survival mechanisms and protect the myocardium against ischemia and reperfusion injury. For example, in animal models, administration of LPS or its non-pyrogenic derivative mono-phosphoryl lipid A reduces cardiac arrhythmia and myocardial infarction after ischemia and reperfusion injury (IRI) (23–27). In a rabbit model systemic administration of LPS before ischemia led to a reduction in infarct size by 54% (28). Using an ex vivo Langendorff model, Meng et al. (29) demonstrated that prior systemic treatment of rats with LPS led to an improved cardiac function during reperfusion phase after transient ischemic insult as compared with the control saline-treated rats.

Although the cardio-protective effects of LPS against IRI are well documented, its effect on myocardial apoptosis is unclear. LPS has been shown to activate parallel pro-apoptotic and survival pathways in other cell types. Activation of TLR4 triggers expression of cell survival and inflammatory genes via NF-κB-dependent mechanisms, although in endothelial cells, NF-κB activity seems dispensable for the LPS-induced cytoprotective activity (16). LPS also activates phosphatidylinositol 3-kinase/Akt pathways in some cell types (30, 31). In endothelial cells LPS induces apoptotic signaling through a mechanism involving TRAF-6-mediated c-Jun NH2-terminal kinase activation (32). However, LPS-induced apoptosis is often cell type-specific (most in endothelial cells) and depends on the simultaneous administration of cycloheximide or proteasome inhibitor to block the synthesis of endogenous survival molecules (33, 34). The survival signals in LPS-treated endothelial cells appear to include both an inducible and constitutively active components (34, 35).

In the present study we examined the anti-apoptotic effect of TLR4 activation by LPS in an in vitro model of ischemia, i.e. hypoxia/serum deprivation (SD). LPS pretreatment led to improved survival and function of CMs subjected to SD. Employing transgene expression and pharmacological inhibitors, we explored the roles of IRAK-1 and other three survival molecules, Akt, ERK1/2, and IKKβ in LPS cardio-protective effects. Finally, we demonstrated that IRAK-1, an important component of TLR4 innate signaling, is dynamically activated in CMs subjected to hypoxia/reoxygenation and in myocardium subjected to coronary artery ligation. These results suggest that the anti-apoptotic effect of LPS-TLR4 signaling may play an important role in protecting CMs and that the myocardial innate immune system involving IRAK-1 may represent an intrinsic mechanism of cardio-protection.

EXPERIMENTAL PROCEDURES

Materials—LPS (Escherichia coli 0111:B4), collagenase, myelin basic protein, and β-actin antibody were from Sigma. Antibodies for cleaved caspase-3, Akt, P-Akt, and P-ERK were from Cell signaling (Beverly, MA), and antibodies for IRAK-1 were from Pro-Sci Co. (catalog number 1007) and Santa Cruz (F4, sc-5288), respectively. Wortmannin and PD98059 were from Calbiochem. Cell death detection ELISA kits were from R&D Systems. Caspase-3 activity assays kits were from R&D Systems.

Neonatal Rat CM Preparation—CMs were prepared from 1–2-day-old rats using collagenase and pancreatic. Briefly, the hearts were isolated, dissected from major vessels, and cut into small pieces. The heart tissues were then incubated in ADS buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM NaH2PO4, 5.6 mM glucose, 5.4 mM KCl, and 0.5 mM MgSO4) containing 0.04% collagenase and 0.06% pancreatic (Worthington) at 37 °C for 20 min in a shaker. Cell suspension was slowly removed, and the remaining myocardial tissues were further incubated with the enzyme buffer. Cells in suspension were collected, spun, and resuspended in Dulbecco’s modified Eagle’s medium containing 10% horse serum, 5% fetal bovine serum, and 4.5% d-glucose. Fibroblasts were then plated in Petri dishes and incubated in CO2 incubator at 37 °C for 3–4 days before experiments were performed.

Adult Rat CM Isolation—Isolated adult ventricular cardiomyocytes were prepared using an enzymatic perfusion method as described by Martin et al. (36). Briefly, the heart was perfused with an enzyme solution containing 0.08% collagenase Type II (Worthington) and 0.04% hyaluronidase (Sigma) in oxygenated Ca2+-free Krebs-Henseleit solution. The heart was perfused for 25 min, and the resulting cell suspension was filtered and triturated with Krebs-Henseleit solution containing 0.5 mM Ca2+ for 5 min. The cells were spun down at 1000 rpm for 1 min and then resuspended in Krebs-Henseleit buffer containing 1.0 mM Ca2+. Adult CMs were incubated in RPMI 1640 with 10% horse serum and 5% fetal bovine serum.

In Vitro Model of Apoptosis and LPS Treatment—Three days after plating, cultured beating neonatal CMs were washed 3 times with serum-free RPMI 1640 medium containing 0.05% bovine serum albumen and incubated in the extracellular medium. In some experiments cell cultures were then placed in a hypoxia chamber filled with humidified flowing hypoxic gas (95% nitrogen and 5% CO2) at 37 °C for 4–6 h. Our previous studies have demonstrated that SD alone for 4–24 h induces significant caspase activation and DNA fragmentation. Hypoxia leads to only modest additional activation of caspase-3 and DNA laddering in the SD model (37, 38). For LPS experiments, cell cultures were prepared in serum-free medium or LPS and the indicated concentration for 80 min. Cells were then washed three times and then incubated with either regular serum-containing RPMI medium or serum-free medium depending on experimental design. Two doses of fresh LPS were added to cell cultures accordingly. In some experiments LPS was added at the time of SD without preincubation.

Apoptosis/Cell Viability Assays—For DNA ladderings, cells were lysed, and cellular DNA was extracted with phenol/chloroform. 1.5 μg of genomic DNA fragments were treated with RNase. 32P-labeled dCTP was added to cell lyses DNA polymerase, and then separated by electrophoresis in 1.8% agarose gel (37, 38). For cell death ELISA, apoptosis-induced histone DNA fragments were quantitated using cell death detection ELISA (37, 38). For caspase activity assays, caspase-3 activity was examined by using the caspase colorimetric assay kit from R&D Systems as previously described (37). For the MTT assay, neonatal CMs were plated in 24-well plates (6×104/well) were incubated in either regular RPMI medium or serum-free medium as indicated for 6 h. At the end of incubation, 1.5 ml of fresh regular RPMI medium containing 0.05% MTX was added into well each and incubated at 37 °C for 1 h. The MTX-containing medium was then removed. 1.5 ml of Me2SO was added in each well, and absorbance was measured at 570 nm.

Immunoprecipitation, Western Blotting, and IRAK-1 Kinase Assay—CMs (~2×106 cells) were scraped from 60-mm dishes in RPMI 1640 medium, spun down, and washed once with 15 ml of cold phosphate-buffered saline. Cell pellets or freshly frozen rat myocardial tissue (see In Vivo Model of Ischemia Reperfusion) were dissolved in 1 ml of cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 20 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM dithiothretiol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, aprotinin, and pepstatin) and incubated for 10 min at 4 °C. Cell lysate was then centrifuged at 10,000 rpm for 10 min, and supernantant was removed. For immunoprecipitation, 2.5 μg of polyclonal anti-IRAK-1 antibody (Pro-Sci Co.) was added to cell lysates containing equal amount of proteins and incubated at 4 °C for 3 h or overnight on a rotator. To harvest IRAK-1-antibody complex, 50 μl of pre-washed 50% slurry protein G (Amersham Biosciences) was added, incubated for 2 h at 4 °C, spun down at 14,000 rpm in an Eppendorf centrifuge, and washed 4 times with cold lysis buffer. Half of the protein G beads were used for IRAK-1 Western blotting (F4 antibody from Santa Cruz) as described previously (39). The remaining beads were washed twice and incubated in 110 μl of kinase assay buffer (20 mM HEPES, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM EDTA, 1 mM NaVO3, and 1 mM benzamidine). To isolate IRAK-1 kinase assay, 2 μg of myelin basic protein (MBP) (Sigma), 5 μM cold ATP, and 50 μCi of [γ-32P]ATP were added to 110 μl of immunoprecipitates and incubated at 37 °C for 30 min. At the end of the assay, immunoprecipitates were mixed with SDS sample buffer, heated, and separated in 4–20% gradient SDS-PAGE. 32P-labeled MBP was visualized by autoradiography.

Measurement of Ca2+ Transient and Cell Shortening—Adult CMs were loaded with 5 μM Fluo 4-AM (Molecular Probe) at room temperature for 20 min. Cells attached to the coverslip were continuously perfused with Tyrode solution (130 mM NaCl, 5.4 mM KCl, 4 mM NaH2PO4, 0.5 mM MgCl2, 1 mM CaCl2, 25 mM HEPES, 22 mM glucose, 0.01 μg/ml insulin, pH 7.4 with NaOH) and electrically stimulated.
An in vitro model of hypoxia and re-oxygenation was used to mimic in vivo ischemia and involved a brief incubation of CMs in SD and hypoxic conditions. The in vitro model has been demonstrated to induce significant CM apoptosis (37, 38). As shown in Fig. 2A, the combination of hypoxia and serum deprivation for 24 h led to an increase in DNA laddering, and treatment of CMs with LPS at the time of SD substantially inhibited DNA fragmentation. Quantitative analysis using histone-DNA fragment ELISA further demonstrated that hypoxia/SD induced up to an 8.2-fold increase in DNA fragmentation within 9 h in CMs and a 2.4-fold increase in human umbilical vein endothelial cells within 5 h. LPS significantly inhibited hypoxia/SD-induced apoptosis by 45% in cultured rat CMs but failed to inhibit apoptosis in human umbilical vein endothelial cells, suggesting the cyto-protective effect of LPS is cell type-specific (Fig. 2B). To further explore the anti-apoptotic effect of LPS, caspase-3 cleavage and activity were measured. LPS dramatically inhibited the pro-caspase-3 cleavage in a dose-dependent manner (Fig. 2C). Similar to what we documented previously (37), hypoxia/SD induced a time-dependent cleavage and activation of pro-caspase-3, both of which were correlated well with each other. LPS treatment led to a decrease in caspase-3 activity for up to 6 h of SD (Fig. 2, D and E). Consistent with its anti-apoptotic effect, LPS also improved the viability of serum-deprived CMs as demonstrated by MTT assay (Fig. 2F).

LPS Improves Function of Adult CMs Subjected to SD—To learn whether activation of TLR4 innate signaling by LPS improves CM function in an in vitro model of ischemia, cultured adult rat CMs were pre-treated with LPS (500 ng/ml) or saline for 1 h in regular RPMI, washed, and incubated in the presence or absence of serum as indicated. Additional doses of LPS or saline were added after the wash. Five hours later, transients were recorded (Fig. 3A). It is noteworthy that only beating CMs were chosen to record [Ca\(^{2+}\)] transients and cell contractility in all four groups of CMs. Three-dimensional graphics as changes of fluorescence ratio \(\Delta F/F_0\) (where \(F_0\) is the base-line [Ca\(^{2+}\)] signal before depolarization, \(\Delta F = F - F_0\)) is presented in Fig. 3B. The average amplitudes (\(\Delta F/F_0\)) of Ca\(^{2+}\) transients from control CMs (+ serum, saline, n = 36) was 3.1 ± 0.45 (Fig. 3C), the time-to-peak Ca\(^{2+}\) was 138 ± 41 ms (Fig. 3D), the time constant (\(\tau\)) during reuptake and relaxation was 299 ± 65 ms (Fig. 3E), and the cell shortening was 10.1 ± 1.5% (Fig. 3F). Surprisingly, CMs treated with 500 ng/ml LPS in normal serum-containing conditions (+ serum, LPS, 6 h, n = 31) demonstrated improved Ca\(^{2+}\) homeostasis and contractility with a 19% increase in the average amplitudes of Ca\(^{2+}\) (Fig. 3C), a 23% decrease in the time-to-peak Ca\(^{2+}\) (Fig. 3D), and a 35% increase in cell shortening (Fig. 3F) but no significant change in the time constant (Fig. 3E). SD for 5 h dramatically inhibited Ca\(^{2+}\) transients and contractility (Fig. 3, A–F, lower panel). Similar to its CM survival effect, LPS significantly improved the Ca\(^{2+}\) transients and enhanced contractility in living CMs subjected to SD as compared with the saline-treated CMs under the same condition, with a 128% increase in the amplitude of Ca\(^{2+}\) transients (Fig. 3C, lower panel), a 62% reduction in the time constant of [Ca\(^{2+}\)] transients (Fig. 3E, lower panel), and a 136% increase in cell shortening (Fig. 3F, lower panel).

LPS Protective Effect Is Mediated by Multiple Survival Pathways—To identify the downstream signaling pathways mediating the LPS anti-apoptotic effects in CM, we used specific kinase inhibitors (for phosphatidylinositol 3-kinase and MEK1) or transgene expression of IKKβ-dn (41) (to block NF-кB activation) to block the three survival pathways and tested their effect on the LPS-induced anti-apoptotic effect (Fig. 4). In the
absence of LPS, SD (4 h, lane 2) induced increased caspase-3 cleavage, a decrease in P-Akt (Ser-473), and a modest increase in P-ERK. Treatment with LPS under the same conditions (SD) enhanced phosphorylation of Akt/ERKs and inhibited caspase-3 cleavage (lane 6 versus lane 2). Inhibition of phosphatidylinositol 3-kinase and MEK1 by wortmannin and PD98059, respectively, specifically blocked both the baseline and the LPS-induced phosphorylation of Akt and ERKs and reversed the anti-apoptotic effect of LPS as demonstrated by increased caspase-3 cleavage (lanes 3–4 and 7–8) as compared with LPS alone (lanes 2 and 6). Transgene expression of IKK\beta-dn blocked NF-kB activation (data not shown) and abolished LPS-induced inhibition of caspase cleavage despite the up-regulation of phospho-Akt (lane 9). Together, these data suggest that all the three kinases, phosphatidylinositol 3-kinase, ERK, and IKK\beta, contribute to the LPS-induced survival effect in CMs.

IRAK-1 Becomes Activated in Response to Hypoxia/SD in Vitro and Transient Ischemia in Vivo—We hypothesized that TLR4 signaling is dynamically regulated by hypoxic and ischemic insults and represents an intrinsic protective mechanism in the heart. To test the hypothesis, we examined IRAK-1 kinase activity upon hypoxia/SD in cultured CMs and ischemia and reperfusion injury in myocardium. As demonstrated in Fig. 5, combination of hypoxia/SD for 30 min followed by various periods of reoxygenation and serum addition induced a rapid and transient activation of IRAK-1. The activation of IRAK-1 in response to reoxygenation/serum occurred early (10 min) and lasted for 60 min (Fig. 5A). To confirm these in vitro findings, we employed an in vivo rat model to study whether IRAK-1 is activated by myocardial ischemic injury. Healthy rats were subjected to 30 min of coronary artery ligation followed by various periods of reperfusion (Fig. 5B). There was a rapid and robust increase in IRAK-1 kinase activity within 15–45 min after reperfusion started as compared with the sham-operated hearts. To minimize the contributing factor of influx of inflammatory cells during the reperfusion phase, we examined IRAK-1 activity in a straight infarction model without reperfusion. The coronary artery was ligated for 0.5–4 h, and myocardial IRAK-1 activity was examined. As shown in Fig. 5C, myocardial ischemia without reperfusion also induced a dramatic increase in IRAK-1 activity with the peak effect seen at 2 h.

Adenoviral Expression of IRAK-1 Protects CMs against SD-induced Apoptosis—To define the role of IRAK-1 in protecting CM, we have generated adenoviral vectors carrying cDNA for...
FIG. 3. LPS improves [Ca\(^{2+}\)] \(_i\) transients and cell shortening in control CMs and in CMs subjected to SD. A, representative line scan images of Ca\(^{2+}\) transients recorded. The color bar shows the strength of fluorescence signal (F). B, the Ca\(^{2+}\) signal is shown in three-dimensional graphic as changes of fluorescence ratio \(\Delta F/F_0\) (\(F_0\) is the base-line Ca\(^{2+}\) signal before depolarization, \(\Delta F = F - F_0\)). C, average amplitude of [Ca\(^{2+}\)] \(_i\) transients (\(\Delta F/F_0\)) in four groups of CM. D, time-to-peak [Ca\(^{2+}\)] \(_i\). E, average time constant \(\tau\) (ms) of decay of [Ca\(^{2+}\)] \(_i\) transients. F, average cell shortening in % of CM length before depolarization. Experimental conditions: a, CMs in the presence of serum (n = 36); b, CMs in the presence of serum and LPS (500 ng/ml) (n = 31); c, CMs in serum-free medium (n = 30); d, CMs in serum-free medium containing LPS (n = 37). The results were recorded in multiple CMs from four separate adult rat CM preparations. Each data point represents mean ± S.D.
IRAK-1, both wild-type (wt) and a kinase-deficient mutant (K239S). Adenoviral expression of wild-type IRAK-1 resulted in an increase in IRAK-1 kinase activity in the absence of LPS (Fig. 6A, lane 4). LPS treatment led to activation of IRAK-1, and the effect of LPS was further enhanced in the CMs overexpressing wild-type IRAK-1 (Fig. 6A, lane 3 versus 6). Transgene expression of Ad.IRAK-1(K239S) had no kinase activity and did not inhibit LPS-induced IRAK-1 (Fig. 6A) or NF-κB activation (data not shown). It has been observed that IRAK-1(K239S), at a similar level of m.o.i., was consistently expressed at levels much higher than that of IRAK-wt, although no kinase activity was observed. This may be due to the inability of IRAK-1(K239S) to become phosphorylated, which leads to decreased degradation (42). Importantly, adenoviral gene transfer led to m.o.i.-dependent IRAK-1 expression (Fig. 6B, lower panel) and a significant reduction of CM apoptosis induced by SD (Fig. 6, B, upper panel, and C). The protective effect of IRAK-1 was clearly dependent on Ad.IRAK-1 m.o.i., as demonstrated by DNA laddering and by quantitative cell death detection ELISA. In contrast, overexpression of IRAK-1(K239S) failed to protect against SD-induced apoptosis, suggesting an important role of IRAK-1 kinase activity in protecting CMs (Fig. 6, D–E).

**DISCUSSION**

Although LPS is well known for its roles in systemic inflammation and myocardial depression in bacterial sepsis, evidence from several lines of investigation suggests that systemic administration of sublethal doses of LPS protect the myocardium against subsequent ischemia and reperfusion injury (23–27). However, the mechanisms leading to the LPS cardio-protective effect is unknown. The present study was designed to explore the role of LPS and its signaling molecule IRAK-1 in protecting CMs against apoptosis in an in vitro model of ischemia. We found that LPS treatment of CMs led to rapid IRAK-1 activation, decreased apoptosis, and improved overall CM survival. SD induced a dramatic inhibition of $Ca^{2+}$ transients and CM contractility. Similar to its CM survival effect, LPS significantly improved the $[Ca^{2+}]_{i}$ transients and enhanced contractility in surviving and beating CMs subjected to SD. Using specific pharmacological inhibitors and adenovirus-mediated gene transfer, we demonstrated that the survival pathways, phosphatidylinositol 3-kinase/Akt, ERK1/2, and IKKβ, are involved in the LPS anti-apoptotic effects. Moreover, we demonstrated that IRAK-1, an important component of TLR4 innate signaling, is dynamically activated in neonatal CMs subjected to hypoxia/re-oxygenation and in adult myocardium subjected to acute ischemic injury. Overexpression of IRAK-1 but not the kinase-deficient mutant IRAK-1(K239S) led to a significant reduction in CM apoptosis. Together, these results demonstrate an important role of TLR4 activation by LPS in protecting CMs against hypoxia/SD and improving their functions and suggest that IRAK-1-mediated mechanisms may represent an intrinsic cardio-protective mechanism.

The roles of LPS-TLR4 in cell survival and death are complicated. LPS has been shown to activate parallel pro-apoptotic and survival pathways. In most cells activation of TLR4 trig- ers expression of cell survival and inflammatory genes via NF-κB-dependent mechanisms. Prolonged LPS treatment (16–24 h) activates both pro-apoptotic and anti-apoptotic pathways in the heart (43) and induces very modest CM apoptosis (43, 44). However, the low levels of apoptosis appear insuffi-
Ad.GFP–CMs were infected with the control virus activated in LPS-stimulated CMs. Cultured CMs were infected with the control virus Ad.GFP-β-gal (m.o.i. 40), Ad.IRAK-1-wt (m.o.i. 100), or Ad.IRAK(K239S) mutant (m.o.i. 140) overnight. Cells were washed and incubated in serum-free medium for 30 min before LPS (500 ng/ml) stimulation for the indicated times. Cells were lysed and immunoprecipitated for IRAK-1. The immunoprecipitates were assayed for IRAK-1 kinase activity (upper panel) or IRAK-1 Western blotting (WB, lower panel). B—C, adenoviral expression of IRAK-1 inhibits SD-induced CM apoptosis. Neonatal rat CMs were infected with Ad.GFP or Ad.IRAK-1 at various m.o.i. for 14–18 h. IRAK-1 expression at various m.o.i. was confirmed by Western blot (B, lower panel). CMs were washed and incubated in the presence or absence of serum for 5 h. CM apoptosis was then assessed using DNA laddering (B, upper panel) and ELISA for histone-associated DNA fragments (C). C, each data point represents the mean ± S.D. of four separated experiments. Each experiment was performed in duplicate. D–E, adenoviral expression of IRAK-1(K239S) failed to inhibit SD-induced apoptosis. D, upper panel, DNA laddering. Ad.IGF-1 was used as a positive control of anti-apoptotic agent. IRAK-1(K239S) expression was confirmed by Western blot (D, lower panel). E, ELISA for histone-associated DNA fragments. Each data point represents mean ± S.D. of triplicate measurements, and three separate experiments were performed with similar results. For DNA laddering experiments, 60-mm cultures of CMs were used, and at least three separate CM preparations and experiments were performed with similar results.

The present study is the first to our knowledge to demonstrate that brief treatment with LPS improves survival and cellular functions of CMs in an in vitro model of ischemia and that in the absence of pathogen recognition IRAK-1 is dynamically activated by myocardial ischemia and sufficient to confer a CM protective effect. Although it is speculated that TLR4 mediates the direct cardiomyocyte-protective effect of LPS and the activation of IRAK-1 in response to acute ischemia, the exact mechanisms remain to be investigated. Studies employing the mouse CM model genetically lacking TLR4 would be of help to define its roles and are being actively pursued in the laboratory. In addition, several endogenous mediators such as heat shock proteins 60/70 are produced in response to ischemia (49–51) and have been shown to activate TLR4 receptor (52–56). These mediators may play a role in mediating the innate immune signaling in response to acute myocardial ischemia. Consistent with the present finding that innate immune signaling IRAK-1 is activated by myocardial ischemia, a previous study has shown myocardial TLR4 is elevated in murine myocardium of ischemic injury. It is hypothesized that increased expression and signaling by TLR4 may contribute to the activation of innate immunity in injured myocardium (19). However, in vivo implication of TLR4 activation in the myocardium of ischemia and reperfusion injury where there is significant myocardial inflammation may prove complicated. A recent study demonstrates that in TLR4-deficient mice, there is reduced tissue inflammation and myocardial infarction as compared with wild-type animals (57). It is unclear whether the observed cardio-protection is secondary to the impaired cellular functions of TLR4-lacking neutrophils and/or macrophages in these mice.

The mechanisms of LPS effects on myocardial contractile function are not fully understood. In response to LPS, there is increased expression of pro-inflammatory cytokines such as TNFα and interleukin-1β in adult myocardium (58). These pro-inflammatory mediators play critical roles in mediating endotoxin-induced cardiac depression in vivo (59–62). Direct effects of LPS on cardiomyocyte function are much less clear.
Consistent with a previous report in a mouse CM model (63), the present study demonstrates that LPS treatment did not inhibit CM shortening but instead led to enhanced Ca\(^{2+}\) transients and cell shortening under conditions where normal serum-containing medium was used and minimal cell apoptosis occurred. These data suggest that the LPS effect on the CM functions is independent of its survival mechanisms. Furthermore, in CMs subjected to SD, LPS also improved CM function. The degree of functional improvement is not merely the collective results of improved CM survival because Ca\(^{2+}\) transients were recorded in live and beating CMs. Therefore, other mechanisms independent of survival signaling should also be considered. Different from our current findings, previous studies have shown the inhibitory effects of LPS on CM function. In rabbit CM, LPS (1 mg/ml) incubation for 6 h has been shown to induce a modest inhibition (<10%) of cell shortening without change in calcium transient (64). In guinea pig CM, LPS exhibits more significant inhibition on CM contractile function (65). Although it is difficult to explain the discrepancy, it seems reasonable to speculate that several factors such as species, LPS concentration and exposure time, and serum in the assay media may contribute to the differing results. The CM function assays in the above cited studies were performed in serum-free Tyrodes buffer, whereas serum-containing culture medium was employed in our present study. As our and previous studies have documented, serum provides important survival and functional benefits to cultured CMs and serum deprivation, even for a short period of time (4–5 h) and can lead to significant depression of CM function (Ca\(^{2+}\) transients and CM shortening) and cell death.

The all three survival pathways, phosphatidylinositol 3-kinase/Akt (2, 66), ERK1/2 (67), and IKK\(\alpha\) (22004), are present in the heart and play critical roles in protecting myocardium against acute ischemia. LPS has been shown to activate these kinase pathways in various cell types. In B cells, LPS activates the phosphatidylinositol 3-kinase/Akt pathway via TAK1- and receptor-interacting protein-dependent mechanisms (30, 31), which is essential for B cell survival in response to LPS (31). The same pathway mediates LPS-induced endothelial cell survival through a TRAF-6-dependent mechanism (16). ERK1/2 is activated by LPS in macrophages and endothelial cells (15, 16), but its role in LPS-mediated cell survival is unclear (16). Although it remains to be elucidated how these three survival pathways are activated in CMs in response to LPS, our data demonstrated that inhibition of these pathways reverses the anti-apoptotic effect of LPS and suggests the importance of these kinases in mediating LPS-induced CM protection against SD. It is noteworthy that in some cell types, the phosphatidylinositol 3-kinase/Akt pathway interconnects with IKK\(\alpha\)-NF-kB pathway (68, 69). Akt mediates TNF-induced phosphorylation of IKK\(\alpha\) and subsequent NF-kB activation (68). Our data that transgene expression of IKK\(\alpha\)-dn up-regulates phospho-Akt is interesting. It remains unclear whether this is a direct effect or occurs through an intermediary. The effect can be inhibited by wortmannin (data not shown), suggesting that phosphatidylinositol 3-kinase activation is required. Further investigation will be needed to elucidate the mechanism of Akt phosphorylation and its biological significance.

In conclusion, the present study demonstrates that LPS activates the TLR4-signaling pathway in CM, protects CMs against hypoxia/SD-induced apoptosis via multiple downstream survival pathways, and improve cellular functions of CMs. We show that innate signaling via IRAK-1 is dynamically activated in response to acute ischemic injury and that overexpressing IRAK-1 signaling is sufficient to protect CMs against SD-induced cell death.

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