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_J Immunol_ 2007; 178:7317-7324; doi: 10.4049/jimmunol.178.11.7317

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Protection against Myocardial Ischemia/Reperfusion Injury in TLR4-Deficient Mice Is Mediated through a Phosphoinositide 3-Kinase-Dependent Mechanism

Fang Hua,* Tuanzhu Ha,* Jing Ma,* Yan Li,* Jim Kelley,† Xiang Gao,‡ I. William Browder,* Race L. Kao,* David L. Williams,* and Chuanfu Li2*

TLRs play a critical role in the induction of innate and adaptive immunity. However, TLRs have also been reported to mediate the pathophysiology of organ damage following ischemia/reperfusion (I/R) injury. We have reported that TLR4−/− mice show decreased myocardial injury following I/R; however, the protective mechanisms have not been elucidated. We examined the role of the PI3K/Akt signaling pathway in TLR4−/− cardioprotection following I/R injury. TLR4−/− and age-matched wild-type (WT) mice were subjected to myocardial ischemia for 45 min, followed by reperfusion for 4 h. Pharmacologic inhibitors of PI3K (wortmannin or LY294002) were administered 1 h before myocardial I/R. Myocardial infarct size/area at risk was reduced by 51.2% in TLR4−/− vs WT mice. Cardiac myocyte apoptosis was also increased in WT vs TLR4−/− mice following I/R. Pharmacologic blockade of PI3K abrogated myocardial protection in TLR4−/− mice following I/R. Specifically, heart infarct size/area at risk was increased by 98% in wortmannin and 101% in LY294002-treated TLR4−/− mice, when compared with control TLR4−/− mice. These data indicate that protection against myocardial I/R injury in TLR4−/− mice is mediated through a PI3K/Akt-dependent mechanism. The mechanisms by which PI3K/Akt are increased in the TLR4−/− myocardium may involve increased phosphorylation/inactivation of myocardial phosphatase and tensin homolog deleted on chromosome 10 as well as increased phosphorylation/inactivation of myocardial glycogen synthase kinase-3β. These data implicate innate immune signaling pathways in the pathobiology of acute myocardial I/R injury. These data also suggest that modulation of TLR4/PI3K/Akt-dependent signaling pathways may be a viable strategy for reducing myocardial I/R injury. The Journal of Immunology, 2007, 178: 7317–7324.

According to the National Health and Nutrition Examination Survey (1999–2002), Centers for Disease Control and Prevention/National Center for Health Statistics, >490,000 Americans die from myocardial infarction each year and more than a million new cases of ischemic heart disease will occur this year. Despite extensive investigation, we still do not fully understand the cellular and molecular mechanisms that are involved in the initiation and propagation of myocardial injury in response to ischemia/reperfusion (I/R)3 injury; nor do we understand the innate physiologic mechanisms that attempt to limit inflammation, maintain homeostasis, and promote survival of the heart in I/R. TLRs are pattern recognition receptors that play an important role in the induction of innate immunity by recognition of exogenous pathogen-associated molecular patterns and endogenous ligands (1). TLR-mediated signaling mainly activates intracellular signaling pathways, such as NF-κB, which plays a critical role in regulating expression of genes that are involved in innate immunity and inflammatory responses as well as cell growth, cell survival, and cell death (2). TLR4 recognizes Gram-negative bacterial LPS (1) and endogenous ligands such as heat shock proteins 60 and 70 (3). Innate immune and inflammatory pathways have been implicated in myocardial I/R injury and congestive heart failure (4, 5). Recent evidence suggests that TLR4-mediated NF-κB activation plays an important role in myocardial I/R injury (5–13). Blunting NF-κB activation significantly reduces myocardial injury following I/R, improves cardiac functional recovery (5, 9, 10), and down-regulates inflammatory cytokine and adhesion molecule gene expression (14). Increased TLR4 expression has been observed in human heart failure and ischemic hearts (11). TLR4 also plays a role in myocardial dysfunction during bacterial sepsis (15) and pressure overload-induced cardiac hypertrophy (16). We have reported that modulation of TLR4-mediated signaling rapidly induces cardioprotection in a rodent model of I/R injury (5). Collectively, these data implicate TLR4/NF-κB-mediated signaling in the pathobiology of ischemic heart injury. However, the mechanisms by which modulation of TLR4/NF-κB-mediated signaling protects the myocardium from I/R injury are unclear.

Recent evidence has identified cross-talk between TLR signaling and the PI3K/Akt pathway (17, 18). Indeed, TLR stimulation induces activation of the PI3K/Akt pathway (18, 19). The PI3Ks and their downstream target serine/threonine kinase Akt (also

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Received for publication August 10, 2006. Accepted for publication March 13, 2007.

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1 This work was supported in part by National Institutes of Health R01 HL071837 to T.H.; a fellowship grant (to X.G.); and National Gongguan Project of China (NGGPOC) GM53552 (to D.L.W.); East Tennessee State University Research Development (C.L.). This work was also supported in part by National Institutes of Health R01 HL071837 to T.H.; and National Gongguan Project of China (NGGPOC) GM53552 (to D.L.W.); East Tennessee State University Research Development (C.L.).

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3 Abbreviations used in this paper: I/R, ischemia/reperfusion; GSK, glycogen synthase kinase; IA/RA, infarct area vs risk area; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RA/LV, risk area vs left ventricle; TTC, triphenyltetrazolium chloride; WT, wild type.

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known as protein kinase B) are a conserved family of signal transduction enzymes that are involved in regulating cellular activation, inflammatory responses, chemotaxis, and apoptosis (20). We (5, 21, 22) and others (23–25) have reported that the PI3K/Akt signaling pathway may be an endogenous negative feedback regulator or compensatory mechanism that serves to limit proinflammatory and apoptotic events in response to injurious stimuli. Indeed, activation of PI3K/Akt-dependent signaling has been shown to prevent cardiac myocyte apoptosis and protect the myocardium from I/R injury (26–29). We have also shown that activation of the PI3K/Akt signaling pathway is associated with decreased myocardial ischemic injury through modulation of TLR4-mediated signaling (5). In addition, basal levels of phosphorylated Akt in the myocardium of TLR4-deficient (TLR4–/–) mice, subjected to pressure overload, are higher than those observed in wild-type (WT) mice (16). We hypothesized that PI3K/Akt-dependent signaling may play a role in the cardioprotection of TLR4-deficient mice following I/R. To test this hypothesis, we pharmacologically inhibited PI3K/Akt-dependent signaling in TLR4–/– mice before myocardial I/R injury and then examined myocardial infarct area vs risk area (IA/RA) and cardiac myocyte apoptosis.

Materials and Methods

Experimental animals

The murine strain C3H-Tlr4–/– on a BALB/c background does not express functional TLR4 due to naturally occurring mutations in the TLR4 gene. WT BALB/c mice served as the genetic background control. TLR4-deleted strain (C57BL/10ScCr) and WT control (C57BL/10ScSn) were also used in the present study (16). All strains of mice were purchased from The Jackson Laboratory and maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this study conformed with the “Guidelines for the Care and Use of Laboratory Animals.” All aspects of the animal care and experimental protocols were approved by the East Tennessee State University Committee on Animal Care.

Experimental model of myocardial I/R injury

We used a rodent model of myocardial I/R injury that mimics the clinical scenario of myocardial infarction (7). Briefly, the mice were anesthetized by isoflurane inhalation and ventilated with room air using a rodent ventilator. After left thoracotomy and exposure of the hearts, the left anterior descending coronary artery was ligated with 6-0 silk ligature just proximal to its main branching point. The suture was tied using a shoestring knot over a 1-mm polyethylene tube (PE-10) that was left in place during the planned period of ischemia. Myocardial ischemia was confirmed by S-T elevation and by isoflurane inhalation and ventilated with room air using a rodent ventilator. After left thoracotomy and exposure of the hearts, the left anterior descending coronary artery was ligated with 6-0 silk ligature just proximal to its main branching point. The suture was tied using a shoestring knot over a 1-mm polyethylene tube (PE-10) that was left in place during the planned period of ischemia. Myocardial ischemia was confirmed by S-T elevation.

Isolation of cellular protein from ischemic and nonischemic myocardium

To examine the effects of PI3K/Akt inhibition on cellular signaling following myocardial I/R, TLR4–/– and WT control mice were treated with or without wortmannin 1 h before I/R injury. Sham surgery mice treated with or without wortmannin served as control. The hearts were harvested at the end of the reperfusion period and the blood was washed out with ice-cold PBS. The right ventricle and atria were trimmed away and the left ventricle was divided into ischemic and nonischemic zones, based on the anatomical landmarks of the cyanotic and bulging region (5, 7). Cellular proteins were isolated from ischemic and nonischemic regions of the myocardium, as described previously (5, 7).

Determination of myocardial infarct size

Infarct size was established by triphenyltetrazolium chloride (TTC; Sigma-Aldrich) staining, as described previously (5, 7). Briefly, the hearts were removed and perfused with saline on a Langendorff system to wash blood from the coronary vasculature, followed by staining with 1% Evans blue to determine the area at risk. Each heart was then sliced horizontally to yield five slices. The slices were incubated in 1% TTC prepared with 200 mM Tris buffer (pH 7.8) for 15 min at 37°C. Viable nonischemic myocardium stains blue with TTC. Ischemic myocardium, which is still viable, stains red with TTC, whereas the necrotic myocardium does not stain and appears pale white. The infarct area (white) and the area at risk (red and white) from each section were measured using an image analyzer. Ratios of risk area vs left ventricle (RA/LV) and IA/RA were calculated and expressed as a percentage.

In situ apoptosis assay

In situ cardiac myocyte apoptosis was examined by the TUNEL assay (Boehringer Mannheim), as described previously (5, 16). Hearts were sectioned and embedded in paraffin. Three slides from each block were evaluated for percentage of apoptotic cells using the TUNEL assay. Four slide fields were randomly examined using a defined rectangular field area with magnification ×200. One hundred cells were counted in each field, and apoptotic cardiac myocytes were presented as the percentage of total cells counted.

Immunohistochemistry for activated caspase-3 and phospho-phosphatase and tensin homolog deleted on chromosome 10 (PTEN)

Immunohistochemistry was performed to examine caspase-3 activity and phospho-PTEN in the heart sections using specific anti-caspase-3-cleaved Ab or anti-phospho-PTEN Ab, respectively (Cell Signaling Technology), as previously described (21). Briefly, hearts from each group were harvested and immersion fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut at 5 μm, and stained with an Ab directed against activated caspase-3 or phospho-PTEN (21). Three slides from each block were evaluated with brightfield microscopy.

EMSA

Nuclear proteins were isolated from heart samples, as previously described (5–8, 16). NF-κB-binding activity was examined by EMSA in a 15-μl binding reaction mixture containing 15 μg of nuclear proteins and 35 fmol of [γ-32P]labeled dsNF-κB consensus oligonucleotide.

Western blots

Cytoplasmic proteins were prepared from heart tissues, and immunoblots were performed, as described previously (5–8, 16). Briefly, the cellular proteins were separated by SDS-PAGE and transferred onto Hybond ECL membranes (Amersham Biosciences). The ECL membranes were incubated with the appropriate primary Ab, i.e., anti-phospho-Akt (Thr308), anti-phospho-glycogen synthase kinase (GSK) 3β, anti-phospho-PTEN (Cell Signaling Technology), anti-GSK-3β, anti-Akt, or anti-PTEN (Santa Cruz Biotechnology), respectively, followed by incubation with peroxidase-conjugated secondary Abs (Cell Signaling Technology). The signals were detected with the ECL system (Amersham Biosciences). To control for loading, the same membranes were probed with anti-GAPDH (BioDesign) after being washed with stripping buffer. The signals were quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad). The results from each experimental group were expressed as relative integrated intensity compared with that of control hearts measured with the same batch.
5.67%)-treated TLR4 in both wortmannin (39.51/H11006) I/R injury (Fig. 2A) may be responsible for protection of the myocardium from I/R injury (5). To investigate whether increased activation of the PI3K/Akt signaling pathway protects the myocardium from I/R injury (32–34). Activation of the PI3K/Akt pathway may be responsible for protection of the myocardium from I/R injury in TLR4−/− mice, we administered the PI3K inhibitors, wortmannin or LY294002 to WT mice. After 1 h before I/R. In agreement with previous data, Fig. 1A shows that I/R induced an IA/RA (index for myocardial injury) of 40.6 ± 2.78% in WT (C57BL/10ScSn) mice vs 19.2 ± 2.79% in TLR4−/− mice (C57BL/10ScCr). This is a 51.2% reduction in I/R-induced myocardial injury in TLR4−/− mice compared with WT mice. The difference in infarct size can be clearly seen in a representative sectioned heart shown on the right of Fig. 1. Heart sections from a WT mouse, subjected to I/R, show little evidence of infarction or necrotic myocardium (Fig. 1). There is no significant difference in RA/LV, which reflects the position of coronary artery ligation, between TLR4−/− mice and WT mice. Similar results were observed in the other strain of TLR4-deficient mice (C57BL/10ScCr). This can be clearly seen in the heart sections on the right of Fig. 1B. Hearts from mice treated with wortmannin or LY294002 show large infarct areas (white, no TTC staining), when compared with heart sections from a TLR4−/− mouse that was not treated with PI3K inhibitors. Administration of wortmannin or LY294002 to WT mice did not alter I/R-induced IA/RA (Fig. 2B).

PI3K inhibition with wortmannin or LY294002 increased cardiac myocyte apoptosis in TLR4−/− mice following I/R

Cardiac myocyte apoptosis plays a major role in cardiac dysfunction following myocardial I/R injury (32–34). Activation of the PI3K/Akt pathway decreases cardiac myocyte apoptosis following myocardial I/R (26–29). Therefore, we examined cardiac myocyte apoptosis in WT and TLR4−/− mice following I/R injury using two approaches, i.e., the TUNEL assay and immunohistochemistry of activated caspase-3. Fig. 3 shows that I/R induced cardiac myocyte apoptosis in WT mice (23.1 ± 1.9%). In contrast, TLR4−/− mice showed only 5.94 ± 1.76% myocyte apoptosis in response to I/R. This is a 74.2% reduction in cardiac myocyte apoptosis when compared with WT mice. Interestingly, wortmannin administration increased myocardial apoptosis in both control and TLR4−/− myocardium. Similar results were observed for LY294002 administration (data not shown).

Activation of caspase-3 is an established marker for apoptotic cells. We examined caspase-3 activity by immunohistochemistry with cleaved caspase-3 Ab. As shown in Fig. 4, caspase-3 activity was increased in the myocardium of WT mice in response to I/R, when compared with sham surgery controls (top left). In contrast, TLR4−/− mice did not show a significant increase in myocardial caspase-3 activity following I/R (bottom left). Inhibition of PI3K with wortmannin resulted in significant increases in myocardial caspase-3 activity in both WT and TLR4−/− mice in response to I/R, although the magnitude of the increase was significantly greater in the WT mice (Fig. 4). Interestingly, administration of wortmannin to WT mice increased cardiac myocyte apoptosis and caspase-3 activity in the sham controls even in the absence of I/R (15.62 ± 2.08 vs 5.62 ± 1.30). However, we did not observe a similar effect of wortmannin in TLR4−/− mice in the absence of I/R.

**Statistical analysis**

Data are expressed as mean ± SE. Comparisons of data between groups were made using one-way ANOVA, and Tukey’s procedure for multiple range tests was performed. Value of p < 0.05 was considered to be significant.

**Results**

**PI3K inhibition with wortmannin or LY294002 abrogated protection of the myocardium from I/R injury in TLR4−/− mice**

We have reported previously that the levels of phospho-Akt in the myocardium in TLR4−/− sham mice are higher than in WT sham mice (16). We have also reported that activation of the PI3K/Akt signaling pathway protects the myocardium from I/R injury (5). To investigate whether increased activation of the PI3K/Akt pathway may be responsible for protection of the myocardium from I/R injury in TLR4−/− mice, we administered the PI3K inhibitors, wortmannin or LY294002, 1 h before I/R. In agreement with previous data, Fig. 1A shows that I/R induced an IA/RA (index for myocardial injury) of 40.6 ± 2.78% in WT (C57BL/10ScSn) mice vs 19.2 ± 2.79% in TLR4−/− mice (C57BL/10ScCr). This is a 51.2% reduction in I/R-induced myocardial injury in TLR4−/− mice compared with WT mice. The difference in infarct size can be clearly seen in a representative sectioned heart shown on the right of Fig. 1. Heart sections from a WT mouse, subjected to I/R, show a large necrotic infarct area (white, no TTC staining). In striking contrast, heart sections from a TLR4−/− I/R mouse show little evidence of infarction or necrotic myocardium (Fig. 1). There is no significant difference in RA/LV, which reflects the position of coronary artery ligation, between TLR4−/− mice and WT mice. Similar results were observed in the other strain of TLR4-deficient mice (C57BL/10ScCr) and WT control mice (BALB/c background) (Fig. 1B). Pharmacologic inhibition of PI3K with wortmannin or LY294002 abrogated cardioprotection observed in TLR4−/− mice following I/R injury (Fig. 2A). The IA/RA was significantly (p < 0.05) greater in both wortmannin (39.51 ± 5.74%) and LY294002 (40.06 ± 5.67%)-treated TLR4−/− mice, when compared with TLR4−/− mice that did not receive the inhibitors (19.92 ± 2.79%). This can be clearly seen in the heart sections on the right of Fig. 2A. Hearts from mice treated with wortmannin or LY294002 show large infarct areas (white, no TTC staining), when compared with heart sections from a TLR4−/− mouse that was not treated with PI3K inhibitors. Administration of wortmannin or LY294002 to WT mice did not alter I/R-induced IA/RA (Fig. 2B).
PI3K inhibition decreased the levels of myocardial phospho-Akt and phospho-GSK-3β in TLR4−/− mice following I/R injury

We have observed previously that the levels of phospho-Akt in the myocardium of TLR4−/− sham mice are higher than those observed in WT sham mice (16). In the present study, we examined the levels of phospho-Akt (Thr308) in the myocardium of TLR4−/− mice and WT mice in the presence and absence of myocardial I/R and/or PI3K inhibition. As shown in Fig. 5A, the levels of phospho-Akt in the myocardium of TLR4−/− sham surgery mice are increased by 100%, when compared with WT sham surgery mice (0.40 ± 0.04 vs 0.20 ± 0.04). I/R did not increase phospho-Akt levels in WT mice. The levels of phospho-Akt were equivalent in TLR4−/− mice in the presence or absence of I/R. The levels of myocardial phospho-Akt in TLR4−/− mice subjected to I/R were significantly higher (p < 0.05) than in WT I/R mice (0.47 ± 0.04 vs 0.27 ± 0.06). PI3K inhibition with wortmannin significantly reduced the levels of myocardial phospho-Akt in TLR4−/− mice in the presence (0.25 ± 0.03 vs 0.47 ± 0.04) and absence (0.28 ± 0.01 vs 0.40 ± 0.04) of I/R injury (Fig. 5A). Administration of LY294002 to TLR4−/− mice in the presence and absence of I/R also significantly reduced the levels of phospho-Akt in the myocardium (data not shown).

GSK-3β is an important downstream target of the PI3K/Akt signaling pathway (35). Phosphorylation of GSK-3β by PI3K/Akt results in GSK-3β inactivation (35). Martin et al. (35) have described a "regulatory
function for GSK-3β in modulating the inflammatory response. Specifically, these investigators reported that inhibiting GSK-3 activity, via a PI3K/Akt-dependent phosphorylation, suppressed proinflammatory responses (35). We investigated the effect of I/R on phosphorylation of GSK-3β in TLR4−/− mice, in the presence and absence of PI3K inhibition. Fig. 5B shows that the myocardial levels of phospho-GSK-3β are significantly (p < 0.05) higher in TLR4−/− vs WT mice in the absence (0.62 ± 0.03 vs 0.36 ± 0.09) or presence (0.65 ± 0.10 vs 0.37 ± 0.03) of I/R. I/R in TLR4−/− mice did not change myocardial levels of phospho-GSK-3β relative to the sham surgery controls. Levels of phospho-GSK-3β were significantly reduced following administration of wortmannin in both the sham surgery controls (0.40 ± 0.02 vs 0.62 ± 0.03) and the I/R (0.38 ± 0.06 vs 0.65 ± 0.10) groups (Fig. 5B). Similar results were observed in TLR4−/− mice treated with LY294002 either in the presence or absence of I/R (data not shown).

![FIGURE 4](image)

**FIGURE 4.** TLR4−/− mice show decreased myocardial-activated caspase-3 in response to I/R injury. The harvested heart samples were examined for caspase-3 activity by immunohistochemistry with specific anti-cleaved caspase-3 Ab. Caspase-3 activity in the cardiac myocytes is indicated by brown color staining. Wort = wortmannin.

![FIGURE 5](image)

**FIGURE 5.** Wortmannin administration reduces the levels of phosphorylated Akt and phosphorylated GSK-3β in TLR4−/− mice. TLR4−/− mice (n = 6) and WT (n = 6) were subjected to ischemia (45 min) and reperfusion (4 h). In a separate experiment, TLR4−/− mice (n = 6) and WT mice (n = 6) were treated with wortmannin (25 µg/25 g body weight) 1 h before the hearts were subjected to ischemia (45 min), followed by reperfusion for 4 h. Sham surgical-operated mice served as controls (n = 6 in each group). The hearts were harvested and cytoplasmic proteins were isolated. The levels of phospho-Akt (A) and phospho-GSK-3β (B) were examined by Western blot with specific Abs. Representative results of phospho-Akt and total Akt, and phospho-GSK-3β and total GSK-3β immunoblotting are shown at the top of each pane. * p < 0.05 compared with indicated groups. S = sham; W = wortmannin.
Phospho-PTEN in TLR4/H11006

0.31

significantly higher than that observed in WT mice (0.43/H11006

0.06). I/R did not significantly alter myocardial levels of phospho-

Akt correlated with alterations in phospho-PTEN expression in TLR4−/− mice. Fig. 6 shows that the levels of phospho-

Akt are low in WT mice. However, TLR4−/− mice showed increased levels of myocardial phospho-PTEN in the presence and absence of I/R. Western blot results showed that the levels of phospho-

Akt in the myocardium of TLR4−/− mice were significantly higher than that observed in WT mice (0.43 ± 0.03 vs 0.31 ± 0.06). I/R did not significantly alter myocardial levels of phospho-PTEN in TLR4−/− or WT mice. In addition, inhibition of PI3K by wortmannin or LY294002 did not alter the levels of phospho-

PTEN in the experimental mice.

Pharmacologic inhibition of PI3K does not alter myocardial NF-κB-binding activity following I/R

Activation of myocardial NF-κB has been reported to play an important role in the pathophysiology of myocardial I/R injury (5, 9, 10). Indeed, blunting I/R-induced NF-κB activity attenuates myocardial I/R injury (5, 9, 10). We examined the effect of PI3K inhibition on myocardial NF-κB-binding activity in WT and TLR4−/− mice following I/R (Fig. 7). In agreement with previous reports (5, 9, 10), I/R significantly increased myocardial NF-κB-binding activity by 62.0% in WT mice. In contrast, TLR4−/− mice showed attenuated myocardial NF-κB activity (31.1%) in response to I/R, when compared with their respective sham controls (Fig. 7). Administration of wortmannin to the experimental mice did not alter I/R-increased myocardial NF-κB-binding activity in either WT or TLR4−/− mice. Similar results were observed when LY924002 was administered to the experimental mice (data not shown).

Discussion

We have reported previously that mice that are deficient in TLR4 show decreased myocardial injury following I/R, thus implicating a role for TLR4 in the pathology of myocardial I/R injury (12, 13). In the present study, we extended those observations to demonstrate that the PI3K/Akt signaling pathway is up-regulated in TLR4−/− mice (Fig. 5). Of great significance, in this study we show that cardioprotection in TLR4−/− mice is mediated, in part, through a PI3K-dependent mechanism. Specifically, we demonstrated that pharmacologic inhibition of the PI3K/Akt signaling pathway abrogates myocardial protection against I/R injury in TLR4−/− mice (Fig. 2). These data suggest that up-regulation of the PI3K/Akt signaling pathway in TLR4−/− mice plays a prominent role in the cardioprotection observed in TLR4-deficient mice following I/R-induced heart injury.

Previous studies have shown that TLR4-deficient mice exhibit less inflammation (12) as well as decreased myocardial NF-κB and AP-1 activation (13) following I/R injury. It is well established that TLR4-mediated MyD88-dependent signaling activates NF-κB (1). We and others have reported that I/R significantly increases myocardial NF-κB activation (6–8). This observation was confirmed in the present study. Of greater significance, blunting NF-κB activation protects the myocardium from I/R injury, improves cardiac functional recovery, and down-regulates proinflammatory cytokine and adhesion molecule gene expression (5, 9, 10). We have also reported that blockade of MyD88, by myocardial transfection of a dominant-negative MyD88, significantly reduced myocardial ischemic injury (37). Thus, modulation of TLR4/MyD88/ NF-κB-dependent signaling rapidly induces cardioprotection following I/R (5). Collectively, these data suggest that activation of TLR4/MyD88/NF-κB-dependent signaling pathways in myocardial I/R plays a critical role in the pathology of the acute heart disease (14). In the present study, we observed that I/R-induced NF-κB activation is substantially decreased in TLR4−/− mice (Fig. 7). Decreased NF-κB activation in TLR4−/− mice correlated with decreased myocardial infarct size. These data confirm and extend our observation regarding the deleterious role of TLR/NF-

κB in heart injury. Administration of the PI3K inhibitors, wortmannin or LY294002, to mice before myocardial I/R did not affect NF-κB-binding activity in WT or TLR4−/−, indicating that the effect of these drugs was independent of NF-κB activity.

We have reported that blunting TLR4-mediated NF-κB activation, while simultaneously stimulating the PI3K/Akt signaling pathway, rapidly induces cardioprotection in a rodent model of myocardial I/R injury (5). Recent evidence suggests that the PI3K/ Akt signaling pathway may be an endogenous negative feedback regulator of TLR/NF-κB-mediated proinflammatory responses (25, 38). Furthermore, there is cross-talk between TLR signaling and the PI3K/Akt signaling pathway (17, 19, 39). For example, stimulation of TLR2 or TLR4 can result in activation of PI3K/ Akt-dependent signaling (5, 19, 39). Activation of PI3K/Akt-dependent signaling protects cardiac myocytes from I/R injury and inhibits I/R-induced cardiac myocyte apoptosis (26–29). We observed that the levels of phosphorylated Akt in the myocardium of TLR4−/− mice are higher than that observed in age-matched WT control mice (Fig. 5). This is also true for TRL4−/− mice that have undergone sham surgery or mice that are subjected to myocardial I/R. We speculated that high basal levels of myocardial PI3K/Akt activity in TLR4−/− mice may be responsible for the cardioprotection that is observed in TLR4−/− I/R-challenged mice. To test this hypothesis, we administered two structurally different PI3K inhibitors, wortmannin or LY294002, to TLR4−/− mice before myocardial I/R. Pharmacologic inhibition of PI3K/Akt-dependent
signaling abrogated cardioprotection in TLR4−/− mice following I/R injury (Fig. 2). These data are significant because they demonstrate that activation of the PI3K/Akt signaling pathway plays an important role in protecting the myocardium from I/R injury in the absence of TLR4-mediated signaling. These data may also point to a possible strategy for reducing heart injury in acute myocardial infarction. Specifically, down-regulation of TLR4/NF-κB-dependent signaling, while simultaneously stimulating PI3K/Akt-dependent signaling, may decrease the morbidity and mortality associated with myocardial infarction.

It is unclear why PI3K activity and basal levels of phosphorylated Akt are higher in TLR4−/− mice than in WT mice. One possible explanation is that the genetic depletion of TLR4 removes a down-regulatory signal that constitutively suppresses PI3K/Akt activity in normal mice. We tested this hypothesis by examining levels of total and phosphorylated PTEN. PTEN is a tumor suppressor lipid protein phosphatase that tightly regulates PI3K/Akt activity (40). Activation of PI3K produces the second messenger phosphatidylinositol 3,4,5-triphosphate, in cellular membranes, resulting in the translocation of Akt to cellular membranes, where Akt undergoes phosphorylation at Thr308 by phosphoinositide-dependent kinase-1, resulting in Akt activation. PTEN antagonizes the PI3K/Akt pathway by dephosphorylating phosphatidylinositol 3,4,5-triphosphate at its 3′ inositol position, resulting in decreased translocation of Akt to cellular membranes and subsequent down-regulation of PI3K/Akt activation (41). The regulation of PTEN activity is controversial, however, as Gericke et al. (41) have recently indicated that phosphorylation of PTEN may deactivate the enzyme, whereas dephosphorylation activates it. This type of regulation may explain why increased expression of PTEN leads to decreased levels of phosphorylated Akt with a concomitant increase in myocardial apoptosis. In contrast, mutant PTEN or phosphorylated PTEN enhances PI3K/Akt activity, leading to cardiac hypertrophy (42, 43). These data indicate that PTEN is a crucial negative regulator of PI3K/Akt signaling in the heart (44).

We speculated that alternations in PTEN expression and/or phosphorylation might be responsible for increased basal levels of phosphorylated Akt in TLR4−/− mice. We examined the expression of PTEN in the myocardium of TLR4−/− mice. Interestingly, the levels of phosphorylated, i.e., inactive, PTEN in TLR4−/− mice were higher than those observed in WT mice (Fig. 6), although total active PTEN levels were not different between TLR4−/− and WT mice. Increased myocardial PTEN phosphorylation in TLR4−/− mice may explain the increased basal levels of phosphorylated Akt. Specifically, TLR4 deficiency correlates with increased phosphorylation and inactivation of PTEN, thus resulting in higher basal levels of phosphorylated Akt. Additional studies are needed to define the specific interactions and signaling relationships that exist among TLR4, PI3K/Akt, and PTEN signaling in the heart.

In 2005, Martin et al. (35) reported that the PI3K/Akt pathway differentially regulates cytokine production in response to noxious stimuli through phosphorylation and inhibition of GSK-3. Furthermore, they demonstrated that inhibition of GSK-3β protected mice from endotoxic shock (35). These investigators speculated that the PI3K/Akt/GSK3-β pathway “could potentially serve as a therapeutic target against... inflammatory diseases” (35). GSK-3β is a constitutively active enzyme that is a downstream target of PI3K/Akt (35). Phosphorylation of GSK-3β by Akt results in inactivation of the enzyme (35). Dugo et al. (45, 46) have reported that GSK-3β inhibition protects against organ injury and dysfunction in hemorrhagic shock and endotoxemia. We speculated that increased myocardial PI3K/Akt activity in TLR4−/− mice might result in increased phosphorylation and inactivation of GSK-3β. We observed an increased level of phosphorylated GSK-3β in the myocardium of TLR4−/− mice. Treatment with the PI3K inhibitor wortmannin decreased the levels of phosphorylated GSK-3β in TLR4−/− mice in the presence or absence of I/R. Increased myocardial GSK-3β phosphorylation in TLR4−/− mice correlated with reduced cardiac infarct size. When PI3K was pharmacologically inhibited, the levels of phosphorylated GSK-3β decreased and this correlated with loss of cardioprotection. We conclude that elevated myocardial PI3K/Akt levels in TLR4−/− mice result in increased phosphorylation and inactivation of GSK-3β, which plays a role in cardioprotection of these mice.

In the present study, we have shown that protection against myocardial I/R injury in TLR4-deficient mice is mediated through a PI3K/Akt-dependent mechanism. The mechanisms by which PI3K/Akt are increased in the myocardium of TLR4−/− mice may involve increased phosphorylation and inactivation of the PI3K/Akt-negative regulator, PTEN. Furthermore, increased myocardial Akt activity in TLR4−/− mice is associated with increased phosphorylation and inactivation of myocardial GSK-3β, both of which correlate with increased cardioprotection. These data implicate innate immune signaling pathways in the pathophysiology of acute myocardial infarction and I/R injury. These data have also identified an important physiologic mechanism that acts to counter the deleterious effects of TLR4/NF-κB in myocardial injury. In addition, our data reinforce the notion that TLR4 and PI3K/Akt play a physiologic role by counterregulating each other, in that TLR4 appears to negatively regulate PI3K/Akt, whereas PI3K/Akt acts as a negative feedback regulator of TLR4-induced proinflammatory responses. When considered as a whole, these data suggest that modulation of TLR4/PI3K/Akt-dependent signaling pathways may be a viable strategy for reducing myocardial I/R injury.

Disclosures
The authors have no financial conflict of interest.

References


