Reduced cardiac hypertrophy in toll-like receptor 4-deficient mice following pressure overload

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Abstract

Objective: We have previously demonstrated that nuclear factor kappa B (NF\textsubscript{κ}B) activation is needed for the development of cardiac hypertrophy in vivo. NF\textsubscript{κ}B is a downstream transcription factor in the Toll-like receptor (TLR)-mediated signaling pathway; therefore, we investigated a role of TLR4 in cardiac hypertrophy in vivo.

Methods: TLR4-deficient mice (C3H-\textsuperscript{Tlr4}\textsuperscript{−/−}, n = 6), wild-type (WT) genetic background mice (BALB/c, n = 6), TLR4-deleted strain (C57BL/10ScCr, n = 8), and WT controls (C57BL/10ScSn, n = 8) were subjected to aortic banding for 2 weeks. Age-matched surgically operated mice served as controls. In a separate experiment, rapamycin (2 mg/kg, daily) was administered to TLR4-deficient mice and WT mice immediately following aortic banding. The ratio of heart weight/body weight (HW/BW) was calculated, and cardiac myocyte size was examined by FITC-labeled wheat germ agglutinin staining of membranes. NF\textsubscript{κ}B binding activity and the levels of phospho-p70S6K in the myocardium were also examined.

Results: Aortic banding significantly increased the ratio of HW/BW by 33.9% (0.601 ± 0.026 vs. 0.449 ± 0.004) and cell size by 68.4% in WT mice and by 10.00% (0.543 ± 0.011 vs. 0.495 ± 0.005) and by 11.8% in TLR4-deficient mice, respectively, compared with respective sham controls. NF\textsubscript{κ}B binding activity and phospho-p70S6K levels were increased by 182.6% and 115.2% in aortic-banded WT mice and by 78.0% and 162.0% in aortic-banded TLR4-deficient mice compared with respective sham controls. In rapamycin-treated aortic-banded mice, the ratio of HW/BW was increased by 18.0% in WT mice and by 3.5% in TLR4-deficient mice compared with respective sham controls.

Conclusion: Our results demonstrate that TLR4 is a novel receptor contributing to the development of cardiac hypertrophy in vivo and that both the TLR4-mediated pathway and PI3K/Akt/mTOR signaling are involved in the development of cardiac hypertrophy in vivo.

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1. Introduction

Toll-like receptors (TLRs) are pattern recognition receptors that play an important role in induction of innate immunity by recognition of exogenous pathogen associated molecular patterns (PAMPs) and endogenous ligands [1]. TLR-mediated signaling mainly activates nuclear factor Kappa B (NF\textsubscript{κ}B) which plays a critical role in regulation of the expression of groups of genes involved in immune and inflammatory
responses, cell death and survival, and cell growth and cell cycle [2]. TLR4 has been characterized to specifically recognize Gram-negative bacterial lipopolysaccharide (LPS) [1] and endogenous ligands such as heat shock protein 60 (Hsp60) and Hsp70 [3]. Recent evidence has suggested that TLR4 is involved in cardiovascular disease. For example, increased TLR4 expression has been observed in human heart failure and ischemic hearts [4]. TLR4 plays a role in myocardial dysfunction during bacterial sepsis [5] and myocardial injury following ischemia/reperfusion (I/R) [6]. We have previously reported that modulation of TLR4 mediated signaling rapidly induces cardioprotection in an I/R injury model [7].

The TLR mediated signaling pathway may have cross-talk with the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [8,9]. Activation of PI3K/Akt signaling will stimulate NFκB activation [9]. Cardiac specific expression of constitutively active PI3K (caPI3K) or caAkt resulted in cardiac hypertrophy in transgenic mice [10,11]. Mammalian target of rapamycin (mTOR) is an important intermediate in PI3K/Akt signaling and plays a role in cardiac hypertrophy [12,13]. Inhibiting mTOR, however, only partially attenuates cardiac hypertrophy [12,14], indicating that additional signaling pathways may participate in cardiac hypertrophy. NFκB is a critical transcription factor downstream of the TLR4 mediated signaling pathway [1]. Recent studies suggested that NFκB activation is involved in the hypertrophic response of cultured cardiomyocytes [15,16]. We have demonstrated that NFκB activation is needed for the development of cardiac hypertrophy in vivo [17]. Collectively, these data support a role of signaling pathways that lead to NFκB activation in the development of cardiac hypertrophy, but there is no evidence that TLR4 participates in the development of cardiac hypertrophy in vivo.

In the present study, we investigated a role of TLR4 in the development of cardiac hypertrophy and examined whether PI3K/Akt signaling is involved in the TLR4 mediated signaling pathway contributing to the development of cardiac hypertrophy. We observed reduced cardiac hypertrophy in TLR4-deficient mice following pressure overload. Inhibiting mTOR by rapamycin in TLR4-deficient mice resulted in an additional decrease in the development of cardiac hypertrophy. The results suggest, for the first time to our knowledge, that TLR4 is an important receptor that mediates signaling contributing to the development of cardiac hypertrophy in vivo.

2. Experimental procedures

2.1. Experimental animals

The murine strain C3H-Tlr4<sup>−/−</sup> on a Balb/c background, does not express functional TLR4 due to naturally occurring mutations in the TLR4 gene. Wild type (WT) Balb/c mice served as the genetic background control [18]. TLR4 deleted strain (C57BL/10ScCr) and wild type control (C57BL/10ScSn) were also employed in the present study. All strains of mice were purchased from Jackson Laboratory and maintained at the Division of Laboratory Animal Resources at East Tennessee State University (ETSU). The experiments outlined in this manuscript conform with the Guide for the care and use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

2.2. Aortic banding induced cardiac hypertrophy in mice

Mice (8–10 weeks, 20–22 g) were anesthetized, intubated, and ventilated using a rodent ventilator as described previously [17]. The hearts were exposed through a left thoracotomy in the third intercostal space. After the transverse aorta was isolated from between the carotid arteries, a 7-0 silk suture was drawn under the transverse aorta and tied against a 26-gauge needle which was promptly removed [17]. For the sham operation controls, the identical procedure was performed except a suture was not tied around the aorta.

2.3. Experimental protocols

To determine the role of TLR4 in the development of cardiac hypertrophy in vivo, TLR4-deficient mice C3H-Tlr4<sup>−/−</sup> (n = 6) and genetic background WT mice Balb/c (n = 6), TLR4-deleted mice (C57BL/10ScCr) and genetic background control mice (C57BL/10ScSn) were subjected to aortic banding for 2 weeks. To evaluate whether the PI3K/Akt/mTOR signaling pathway is involved in the development of cardiac hypertrophy induced by aortic banding in TLR4 deficient mice, we administered rapamycin (2 mg/kg/day, Sigma, Co), intraperitoneally (i.p.) to TLR4-deficient mice (n = 6) and age-matched WT mice (n = 6) immediately following aortic banding. After 2 weeks of aortic banding, the hearts were harvested and the ratio of heart weight/body weight (HW/BW) was calculated.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from heart samples and NFκB activity was examined by EMSA as previously described [7,19–21] in a 15 μl reaction mixture containing 15 μg of nuclear proteins and 35 fmol of [γ-<sup>32</sup>P]labeled double-stranded NFκB consensus oligonucleotide. A supershift assay using antibodies to P65 and P50 was performed to confirm NFκB binding specificity as previously described [7,19–21].

2.5. Immunoblotting

Myocardial cytoplasmic proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred
onto Hybond ECL membranes (Amersham Pharmacia, Piscataway, NJ) as previously described [7,19–21]. The membranes were incubated with primary anti-phospho-Akt, anti-phospho-p70S6K or anti-IKKβ (Cell Signaling Technology, Inc), respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell signaling Technology, Inc) and analysis by the ECL system (Amersham Pharmacia, Piscataway, NJ). The same membranes were probed with anti-GAPDH (glyceraldehyde-3-phosphatedehydrogenase, Biodesign, Saco, Maine) after being washed with stripping buffer.

2.6. RT-PCR Assay

RT-PCR assays were performed with RNA PCR kits (Perkin-Elmer Cetus, Norwalk, CT) as previously described [7,19–21]. The PCR products of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and GAPDH were analyzed by agarose gel (FMC Products) electrophoresis. All values obtained with the ANP and BNP primers were normalized to the values obtained with the GAPDH primers.

2.7. Histology

Hearts were harvested from TLR4-deficient mice (n = 4) and WT mice (n = 4) 2 weeks after aortic banding and were embedded in paraffin, cut at 5 μm, counterstained with hematoxylin and eosin (H and E) [17]. Myocardial fibrosis was stained by Masson’s Trichrome. The cardiac myocyte size was examined by FITC labeled wheat germ agglutinin staining and left ventricular cardiac myocyte membranes were observed by fluorescent microscopy. Cardiac myocyte size analysis was performed using a phosphor-imager system (Bio-Rad). A value from each heart was calculated.
by use of the measurements of 40–50 cells from an individual heart. The results were expressed as mean values (Mean±SE) from each experimental group.

2.8. *In situ* apoptosis assay

*In situ* cell death examination was performed as previously described [7]. Hearts were sectioned and embedded in paraffin. Three slides from each block were evaluated for percentage of apoptotic cells using the TUNEL (TdT mediated dUTP nick end labeling) assay (Boehringer Mannheim, Indianapolis, IN). Four slide fields were randomly examined using a defined rectangular field area with magnification ×200. One hundred cells were counted in each field.

2.9. *Statistical analysis*

Results are expressed as means± standard errors. For tests of significance between the groups, one-way analysis of variance (ANOVA) and Tukey’s procedure for multiple range tests were performed. *P*<0.05 was considered to be significant.

3. Results

3.1. Reduced aortic banding induced cardiac hypertrophic response in TLR4-deficient mice

Fig. 1(A) shows that aortic banding for 2 weeks significantly increased the ratio of HW/BW by 33.9% in WT mice compared with age-matched sham controls (0.601±0.026 vs 0.449±0.004, *n*=6) and by 10.00% in TLR4-deficient mice compared with age-matched sham controls (0.543±0.011 vs 0.495±0.005). There were no differences in body weight between aortic banding and age-matched sham controls in WT mice (21.94±0.49 g, *n*=6 vs 21.75±0.97 g, *n*=6) and in TLR4-deficient mice (22.86±0.41 g, *n*=6 vs 23.40±0.60 g, *n*=6). TLR4 deleted mice (C57BL/10ScCr) and WT mice (C57BL/10ScSn) were also subjected to aortic banding for 2 weeks. Aortic banding significantly increased the ratio of HW/BW by 29.74% in WT mice (0.637±0.029 vs 0.491±0.021, *n*=8) and by 5.47% in TLR4-deleted mice (0.482±0.010 vs 0.457±0.016, *n*=8).

Fig. 1(B) shows that the cardiac myocyte size of aortic banded WT mice was increased by 68.4% compared with
sham operated control. The cell size of hearts from aortic banded TLR4-deficient mice, however, was only increased by 11.8% compared with age-matched sham operated control.

Fig. 2 shows the levels of ANP and BNP mRNA expression in aortic banded hearts. The levels of ANP (A) and BNP (B) mRNA expression were increased by 230.7% and 106.5% in WT mice (n=6) and by 127.8% and 80.1% in TLR4-deficient mice (n=6), respectively, compared with age-matched respective sham controls.

3.2. Reduced cardiac myocyte death and loss in aortic banded TLR4-deficient mice

Fig. 3 shows histological changes in aortic banded hearts from WT mice (n=4) and from TLR4-deficient mice (n=4). Aortic banding-induced hypertrophic hearts in WT mice exhibited with extensive cardiac myocyte death and loss and increased myocardial interstitial fibrosis (Fig. 3 B, blue on Masson’s trichrome stain). The TUNEL assay showed that the
number of positively stained cardiac myocytes in aortic banded WT mice was significantly increased by 8.61% compared with representative sham control [Fig. 3(C)]. However, there was no significant cardiac myocyte death and loss or myocardial interstitial fibrosis in aortic banded TLR4-deficient mice (Fig. 3(A, B)). Cardiac myocyte apoptosis was increased by 2.61% in aortic banded TLR4-deficient mice compared with age-matched sham control [Fig. 3(C)].

3.3. Blunted myocardial NFκB activation in aortic banded TLR4-deficient mice

Fig. 4(A) shows that NFκB binding activity is low in sham control hearts but significantly increased by 182.6% in the hypertrophic hearts of WT mice and by 78.0% in TLR4-deficient mice compared with age-matched respective sham controls. The specificity of NFκB binding activity in the hypertrophic hearts was confirmed by addition of 100-fold excess of unlabeled NFκB or activated protein II (APII) oligonucleotides into the EMSA reaction. Unlabeled NFκB oligonucleotides competed for the binding of nuclear proteins prepared from aortic banded mouse hearts [Fig. 4(B)], while the AP-II oligonucleotides did not. The predominant protein complex of NFκB containing P50 and P65 subunits in the hypertrophic hearts was demonstrated by antibody supershift assays. Both antibodies, when added separately or together, shifted the major NFκB binding complex [Fig. 4(B)].

3.4. Blunted IKKβ activity in aortic banded TLR4-deficient mice

Fig. 5 shows the levels of IKKβ phosphorylation in aortic banded hearts from WT (n=6) and TLR4-deficient mice (n=6). The levels of phosphorylated IKKβ were significantly increased in the aortic banded hearts by...
199.3% from WT mice and by 156.5% from TLR4 deficient mice compared to the age-matched sham controls (Fig. 5). However, the increase in the levels of IKKβ phosphorylation in aortic banded WT mice was significantly higher than that of TLR4-deficient mice ($P<0.05$).

### 3.5. Activation of Akt in aortic banded TLR-deficient mice

To investigate whether aortic banding can activate PI3K/Akt signaling, TLR4-deficient mice ($n=6$) and WT mice ($n=6$) were subjected to aortic banding for 2 weeks. The levels of phosphorylated Akt were examined by Western blot with a specific antibody. As shown in Fig. 6, the levels of phospho-Akt in aortic banded WT mice were significantly higher than that of aortic banded TLR4 deficient mice ($P<0.05$).

### 3.6. Rapamycin prevents aortic banding-induced cardiac hypertrophy

As shown in Fig. 1, aortic banding can still induce cardiac hypertrophy in TLR4-deficient mice although the degree of hypertrophy is significantly less than that of WT mice. To investigate whether aortic banding-induced cardiac hypertrophy in TLR4-deficient mice is mediated through the PI3K/Akt/mTOR signaling pathway, rapamycin (2 mg/kg/daily) was administered into the TLR4-deficient mice and WT mice immediately following aortic banding. After 2 weeks, hearts were harvested and the ratio of HW/BW was calculated. As shown in Fig. 7(A), in rapamycin treated aortic banded mice, the ratio of HW/BW was increased by 18.0% in WT mice and by 3.5% in TLR4-deficient mice, respectively, compared with respective sham controls. To examine whether rapamycin reduction of the ratio of HW/BW would be associated with decreased NFκB activation, we analyzed NFκB binding activity in the aortic banded hearts. Fig. 7(B) shows that rapamycin treatment significantly reduced NFκB binding activity by 50.2% in WT mice and by 81.7% in TLR4-deficient mice compared with untreated aortic banded respective hearts.

### 3.7. Rapamycin decreased phosphorylated p70S6K in the myocardium following aortic banding

Fig. 8 shows that aortic banding significantly increased the levels of phosphorylated p70S6K in WT mice by 115.2% and in TLR4-deficient mice 162.0% compared with their respective sham controls. Administration of rapamycin significantly reduced the levels of phosphorylated p70S6K by 55.2% in WT mice and by 56.1% in TLR4-deficient mice, compared with their respective age-matched sham controls.

### 4. Discussion

A significant finding of this study is that TLR4-deficient mice showed reduced cardiac hypertrophy after aortic banding in vivo compared with WT control mice, suggesting that TLR4 is an important receptor that mediates signaling pathways contributing to the development of cardiac hypertrophy. We also observed that inhibiting target...
of rapamycin (TOR) by rapamycin completely inhibits aortic banding-induced cardiac hypertrophy in TLR4-deficient mice. This result suggests that both the TLR4 mediated pathway and PI3K/Akt/mTOR signaling participate in the development of cardiac hypertrophy in vivo.

We have previously reported that NFκB activation is needed for the development of cardiac hypertrophy in vivo [17]. We also observed that TLR4 expression was significantly increased in the hypertrophic hearts induced by aortic banding (unpublished data). Since the TLR4 mediated signaling pathway mainly activates NFκB [1], we hypothesized that TLR4 might play a role in the development of cardiac hypertrophy in vivo. To evaluate our hypothesis, we banded the aorta of TLR4-deficient mice and WT genetic background mice for 2 weeks. We observed that aortic banding-induced cardiac hypertrophy was significantly less in TLR4-deficient mice compared with that of WT type genetic background control mice. Similarly, NFκB binding activity in aortic banded hearts of TLR4-deficient mice was also significantly less than that of WT genetic background mice. Our observation suggests that TLR4 mediated signaling could be a novel pathway contributing to cardiac hypertrophy. Recent studies have shown that activation of the signaling intermediates in the TLR mediated NFκB pathway is involved in the hypertrophic response [4,22,23]. For example, increased TLR4 expression was observed in the failing human heart [4]. Transforming growth factor-β activated kinase 1 (TAK1) activity, a key kinase in the TLR mediated NFκB activation pathway, was increased in the heart following aortic banding [22]. Transgenic mice with cardiac specific expression of constitutively active TAK1 developed to cardiac hypertrophy [22]. IKKβ is an important kinase for phosphorylation and degradation of IκBα and activation of NFκB. Inhibiting IKKβ activity prevented hypertrophic growth through inhibition of NFκB activation [15]. Collectively, these data suggest that the TLR4 mediated NFκB activation pathway plays a role in the development of cardiac hypertrophy in vivo.

We have noted that cardiac myocyte death and loss in aortic banded TLR4-deficient mice is less with concom-
evidence suggests that TLR mediated NFκB during the development of cardiac hypertrophy. Recent
activation could be responsible for cardiac myocyte death [24,25]. Bacterial TLR6 with bacterial lipoproteins induces apoptotic cell death [26]. For example, stimulation of TLR2 and TLR6 with bacterial lipoproteins induces apoptotic cell death. For example, myocardial infarction was significantly reduced following I/R in transgenic mice with cardiac specific expression of dominant negative IκBα [27]. Blockade of NFκB activation by decoy oligonucleotides can also protect the heart against I/R injury [28]. Collectively, these data suggest that NFκB plays both anti- and pro-cell death roles after myocardial I/R. We have previously reported that transfection of an adenovirus expressing dominant negative IκBα significantly reduced cardiac myocyte death and loss following 3 weeks of the aortic banding in a rat cardiac hypertrophy model [17]. In the present study, we observed that NFκB activity was reduced, but not completely inhibited, in TLR4-deficient mice after aortic banding compared with that of WT mice. Maintaining certain levels of myocardial NFκB activation, rather than completely inhibiting its activity, may be beneficial for reducing the hypertrophic response of cardiac myocytes without inducing cardiac myocyte apoptosis.

We have noted that the base line of the ratio of HW/BW in sham control of TLR4-deficient mice was higher than that of WT genetic background mice. The mechanisms that result in increased base line of heart weights in TLR4-deficient mice are unknown. No significant changes of phenotype or morphology are observed in TLR4-deficient mice. On the other hand, the degree of cardiac hypertrophy induced by aortic banding in TLR4-deficient mice was significantly less than that of WT genetic background mice, suggesting that TLR4 could play a role in the development of cardiac hypertrophy. The ratio of HW/BW in aortic banded TLR4-deficient mice was also increased compared with sham controls of TLR4-deficient mice, suggesting that additional signaling pathway(s) may participate in the development of cardiac hypertrophy in TLR4-deficient mice. We have previously shown that aortic banding of rat hearts significantly increased the activation of the PI3K/Akt/mTOR signaling pathway [17]. The PI3K/Akt/mTOR pathway has been demonstrated to play a critical role in the development of cardiac hypertrophy [10,11]. In the present study, we observed that the levels of phospho-Akt and phospho-p70S6K were significantly increased in both WT and TLR4 deficient mice. However, the degree of cardiac hypertrophy in TLR4 deficient mice was significantly less than that of wild type mice. To investigate whether the PI3K/Akt/mTOR signaling pathway contributes to cardiac hypertrophy in TLR4-deficient mice following aortic banding, we administered rapamycin to aortic banded TLR4 deficient mice immediately following aortic banding for 2 weeks. Rapamycin is a specific inhibitor for mammalian target of rapamycin (mTOR), which is a component upstream from the ribosomal protein kinase p70S6K in the PI3K/Akt signaling pathway [12] and plays a role in the determination of cell, organ and body size. We have observed that rapamycin administration almost completely inhibited cardiac hypertrophy in TLR4 deficient mice but only partially attenuated cardiac hypertrophy in WT mice. The results suggest that both the TLR4 mediated NFκB activation pathway and PI3K/Akt/mTOR signaling participate in the development of cardiac hypertrophy in vivo.

It is unclear which endogenous ligands that bind to TLR4 contribute to cardiac hypertrophy. Recent evidence indicates that heat shock proteins (Hsps) may be endogenous stimuli for TLRs. For example, Hsp60, Hsp70 and GP96 (Hsp96) interact with TLR2 and TLR4 to activate NFκB [29–31]. Increased levels of Hsp70, TLR2 and TLR4 on monocytes were observed in patients who underwent coronary artery bypass grafting [3], indicating that TLRs might function as key mediators of the immunostimulatory effects of Hsps. Cardiac overload rapidly and transiently induces Hsp70 and Hsp72 mRNA expression [32,33] and overexpression of Hsp56 significantly increased cardiac cell size and hyper-

![Diagram](https://academic.oup.com/cardiovascres/article-abstract/68/2/224/299095)
trophic response in vitro [34]. Although there were controversial reports concerning the contamination of endotoxins in Hsp preparations [35], transgenic mice expressing GP96 triggered the MyD88-dependent NFκB activation pathway [36]. Recombinant GP96 activated dendritic cells independent of LPS but required TLR4 for an optimal cell response [36]. Interestingly, we have observed that pressure overload significantly increased Hsp27 expression (data not shown). Future studies are needed to elucidate the endogenous ligands for TLR4 during the development of cardiac hypertrophy.

In summary, we have observed reduced cardiac hypertrophy in TLR4-deficient mice following aortic banding and that treatment of aortic banded TLR4-deficient mice with rapamycin almost completely prevented aortic banding-induced cardiac hypertrophy. Our results suggest that TLR4 is an important receptor contributing to cardiac hypertrophy and that both the TLR4 mediated NFκB activation pathway and PI3K/Akt/mTOR signaling participate in the development of cardiac hypertrophy in vivo.

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