Modulating Toll-like receptor mediated signaling by (1→3)-β-D-glucan rapidly induces cardioprotection

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Abstract

Objective: Immune and inflammatory signaling pathways, initiated by the innate response, are involved in myocardial ischemia/reperfusion (I/R) injury. Toll-like receptor (TLR) mediated MyD88-dependent NF\textsubscript{k}B pathways play a role in the induction of innate immunity. We have reported that glucan phosphate (GP) improved survival in experimental sepsis, which correlated with decreased tissue NF\textsubscript{k}B activation. In the present study, we report that GP rapidly induced cardioprotection against I/R injury in vivo. Methods: Sprague–Dawley rats were pretreated with GP (40 mg/kg, i.p) 1 h before 45 min of ligation of the left anterior descending coronary followed by reperfusion for 4 and 24 h. Infarction size was examined by triphenyltetrazolium chloride (TTC) staining. NF\textsubscript{k}B activation was analyzed by electrophoretic mobility shift assay (EMSA). I\textsubscript{k}B kinase-\textbeta (IKK\textbeta), IL-1 receptor-associated kinase (IRAK) and Phosphoinositide 3-kinase (PI3K) activities were determined by kinase assay with appropriate substrates. Association of TLR4 with MyD88 or with PI3K p85 was assessed by immunoprecipitation with anti-TLR4 followed by immunoblotting with anti-MyD88 or anti-p85. Results: GP treatment reduced infarct size by 47% in rat hearts subjected to reperfusion for 4 h and by 50% following reperfusion for 24 h. The same protective effect was observed when GP was administrated 5 min after initiation of ischemia. The mechanisms of GP induced cardioprotection involve decreased association of TLR4 with MyD88, inhibition of I/R induced IRAK and IKK\textbeta activity and decreased NF\textsubscript{k}B activity. In addition, GP increased TLR4 phosphotyrosine, resulting in increasing PI3K/Akt activity in the myocardium, which correlated with decreased cardiac myocyte apoptosis following I/R. Conclusion: The results suggest that activation of the TLR mediated MyD88-dependent NF\textsubscript{k}B signaling pathway may play an important role in myocardial I/R injury, while stimulation of the PI3K/Akt signaling could serve a protective role. The data indicates that GP treatment shifts the TLR mediated activation signal in I/R from a predominantly NF\textsubscript{k}B pathway to a predominant PI3K/Akt signaling pathway.

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1. Introduction

Immune and inflammatory pathways, initiated by the innate immune system, have been implicated in myocardial ischemia/reperfusion (I/R) injury and congestive heart failure (CHF) [1]. However, the molecular mechanisms have not been elucidated. Innate immune recognition is mediated by germ-line-encoded receptors that respond to highly conserved macromolecular structures in pathogens called pathogen associated molecular patterns (PAMPs) [2]. Receptors which recognize PAMPs are referred to as pattern-recognition receptors (PRRs). PAMPs include bacterial lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), fungal mannans, and glucans, as well as unmethylated bacterial CpG DNA. Recent studies have highlighted the role of mammalian Toll-like receptors...
(TLRs) in recognition of PAMPs [2]. Toll receptors are an evolutionarily conserved family of signal transducing molecules which are critical for the induction of innate immunity. Mammalian Toll-like receptors (TLRs) have been demonstrated to participate in the response to PAMPs [2,3].

Upon PAMP recognition, TLRs recruit an adapter protein MyD88 to initiate a signaling pathway which is involved in the sequential activation of IL-1 receptor-associated kinases (IRAKs), tumor necrosis factor (TNF) receptor-associated factor 6, nuclear factor Kappa B inducing kinase (NIK), and the IκB kinase complex (IKKs). Activated IKKs lead to IκB phosphorylation and degradation, resulting in nuclear factor KappaB (NFκB) activation [2]. NFκB is a ubiquitous inducible transcription factor which stimulates gene expression, in particular those that promote immune and inflammatory responses [4,5]. The importance of NFκB activation in myocardial I/R has been well documented [6–11]. We have previously demonstrated that I/R rapidly increases myocardial IKKβ activity and IκBα phosphorylation and degradation, resulting in NFκB activation [7–9], suggesting that the TLR mediated MyD88-dependent NFκB signaling pathway may play a role in the inflammatory response to myocardial I/R. Evidence that TLRs play an important role in cardiovascular disease [12–15] has been reviewed recently [16].

Phosphoinositide 3-kinase (PI3K) is a cytoplasmic enzyme which consists of a catalytic subunit, p110, and a regulatory subunit, p85 [17,18]. Serine/threonine kinase Akt (known as protein kinase B) is a well-established target of PI3K [19]. Activation of the PI3K/Akt pathway prevents cardiac myocyte apoptosis and protects the myocardium from I/R injury [17]. The PI3K/Akt pathway can be activated by TLRs through an MyD88-independent pathway [19]. Stimulation of TLR2 and TLR4 or IL-1R results in the recruitment of PI3K to the receptors [19–21], suggesting that stimulation of TLRs not only results in the activation of NFκB through MyD88-dependent pathways, but also activates PI3K/Akt through an alternative pathway that does not signal through MyD88.

Microcellul wall components, such as LPS from gram-negative bacteria and lipoteichoic acid (LTA) from gram-positive bacteria, can induce cardioprotection [22,23] when administered at least 8 h before myocardial I/R. Glucan is a (1 → 3)-β-D-linked polymer of glucose that is isolated from fungal cell walls [24]. Glucan has been extensively studied due to its ability to enhance host innate immunity [25,26]. Clinical and experimental studies suggest that glucan is effective in decreasing septic complications and improving survival in septic hosts [27], which correlates with decreased tissue NFκB activation [25]. In the present study, we demonstrated, for the first time to our best knowledge, that glucan phosphate rapidly induces cardioprotection and does not require pre-treatment in rat hearts subjected to I/R. The mechanisms of protection involves blunting TLR mediated MyD88-dependent NFκB activation and stimulating the TLR mediated MyD88-independent PI3K/Akt pathway.

2. Methods

2.1. Glucan phosphate

Glucan phosphate (GP) is a (1 → 3)-β-D-glucan with a Mw of 1.56 × 10^5 g/mol, which was prepared and characterized in our laboratory [24,28]. The GP is endotoxin-free and is stored as a lyophilized powder (−20 °C).

2.2. In vivo coronary artery occlusion

Male Sprague–Dawley rats (225 to 250 g) were housed in the Division of Laboratory Animal Resources (DLAR) at East Tennessee State University (ETSU). In vivo the left anterior descending (LAD) coronary artery occlusion was performed as previously described [9]. 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). The research protocol was approved by the ETSU Committee on Animal Care.

2.3. Experimental protocols

To investigate the effects of GP on the infarct size/area at risk, rats were pre-treated by intraperitoneal (i.p.) injection with GP (40 mg/kg) 1 h before the hearts were subjected to 45 min of LAD occlusion followed by reperfusion for 4 and 24 h. This dose of GP has been shown to be effective in increasing survival of septic animals [25]. In parallel experiments, GP (40 mg/kg) was administered by intravenous injection (i.v.) 5 min after ischemia. To examine the effects of GP on the TLR mediated MyD88-dependent NFκB pathway and the PI3K/Akt activity, the coronary artery was occluded for 15 min followed by reperfusion for 30 min or 3 h. These time points were chosen because myocardial NFκB is activated during this period [9]. The hearts were immediately harvested and the blood washed out with ice-cold phosphate-buffered saline (PBS). The right ventricle and atria were trimmed away and the left ventricle was divided into ischemic and non-ischemic zones based on the anatomical landmarks of a cyanotic and bulging region for isolation of cytoplasmic and nuclear proteins [9].

2.4. Determination of infarct size/area at risk

The infarct size/area at risk was determined as previously described [29]. Briefly, the hearts were removed and perfused with saline on a Langendorff system to wash blood from the coronary vasculature. After the suture around the
branch of the coronary artery was tied, the hearts were stained with 1% Evans Blue to determine the risk zone. Each heart was then sliced horizontally to yield five slices each approximately 0.2 cm thick. The slices were incubated in 1.5% triphenyltetrazolium chloride (TTC) prepared with 200 mM Tris buffer pH 7.8 for 15 min at 37 °C [29]. Viable myocardium was stained red by TTC, while the necrotic myocardium appeared pale white. The slices were preserved in 10% formaldehyde. The apical side of each slice was imaged and the area of infarction on both sides of each slice were determined by an image analyzer. The area at risk was expressed as a percentage of the left ventricle, and the area of infarction was expressed as a percentage of the area of the tissue at risk.

2.5. Electrophoretic mobility shift assay (EMSA)

NFκB binding activity was examined by EMSA as previously described [7–9] in a 15-μl binding reaction mixture containing, 15 μg of nuclear proteins and 35 fmol of [γ-32P] labeled double-stranded NFκB consensus oligonucleotide. The reaction mixture was separated on 5% non-denaturing polyacrylamide gels and the density of the binding bands was established by densitometric analysis (Genomic Solutions, Ann Arbor, MI). The results from each group were expressed as relative integrated intensity compared to the normal heart group. A supershift assay using antibodies to P65 and P50 was performed to confirm NFκB binding specificity as previously described [7–9].

2.6. Immunoprecipitation

Approximately 200 μg of cellular proteins were immunoprecipitated with 2 μg of antibodies to IRAK, IKKβ or the p85 subunit of PI3K (Santa Cruz Biotechnology, CA) followed by the addition of 15 μl of protein A-agarose beads (Santa Cruz Biotechnology) as previously described [9,26]. Following three times washing, the immunoprecipitates were subjected to kinase activity assay [9,26]. For examination of TLR-MyD88 association, TLR phosphotyrosine and association, the anti-MyD88 immunoprecipitates were probed with anti-phosphotyrosine (p-Tyr 20) and anti-p85 subunit (Santa Cruz Biotechnology), respectively. The results from each group were expressed as relative integrated intensity compared to normal control hearts.

2.7. Kinase activity assay

Following immunoprecipitation, kinase activity was examined with 1 μg of myelin basic protein (MBP, Sigma, St. Louis, MO) for IRAK, GST-IκBα substrate for IKKβ, and 10 μg of sonicated phosphatidylinositol (PI, Sigma) for PI3K and 5 μCi of [γ-32P] ATP (6000 Ci/ mmol, Amersham, Piscataway, NJ) at 30 °C for 30 min as previously described [9,26]. The reaction mixtures were resolved on polyacrylamide gels followed by autoradiography to Kodak X-ray films. The phosphorylation of substrate was quantified by scanning densitometry (Genomic Solutions). For PI3K activity, phosphorylated products were separated on a silica gel thin-layer chromatography plate pre-treated with 1% (wt/vol) potassium oxalate in a chloroform (60 ml)–methanol (47 ml)–water (11.3 ml)–ammonium hydroxide (20–22%, 2 ml) developing solvent. Phosphorylation of substrate was examined by autoradiography and scanning densitometry (Genomic Solutions).

2.8. Immunoblotting

Cytoplasmic proteins (100 μg) or immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, transferred onto Hybond ECL membranes (Amersham), and incubated with IκBα antibody (Santa Cruz Biotechnology) or phospho-Akt antibody (New England BioLabs, Beverly, MA) followed by peroxidase-conjugated second antibodies (New England Biolabs) as previously described [7–9]. The same membranes were probed with anti-GAPDH (glyceraldehyde-3-PDH, Biodesign, Saco, ME) after being washed with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.7). The density of protein signals was scanned (Genomic Solutions). For examination of TLR4-MyD88 association, the anti-MyD88 immunoprecipitates were subjected to immunoblotting with anti-TLR4 antibody. For analysis of TLR4 phosphotyrosine and association with the p85 subunit of PI3K, the anti-TLR4 immunoprecipitates were probed with anti-phosphotyrosine (p-Tyr 20) and anti-p85 subunit (Santa Cruz Biotechnology), respectively. The results from each group were expressed as relative integrated intensity compared to normal control hearts.

2.9. In situ apoptosis assay

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. For the TUNEL negative control, heart tissue sections were incubated with the reaction buffer without terminal transferase. For the TUNEL positive control assay, heart tissue sections were incubated with 1 μg/ml of RNase-free DNase for 10 min at room temperature in order to induce nonspecific breaks in DNA before the TUNEL assay was performed.
2.10. Statistical analysis

Results are expressed as means ± standard errors. For tests of significance between the groups, one-way analysis of variance (ANOVA) was performed. \( P < 0.05 \) was considered significant.

3. Results

3.1. Glucan phosphate reduces infarct size/area at risk

Fig. 1A and B shows that 1 h of pretreatment with GP reduces infarct size/area at risk by 47% after reperfusion for 4 h (25.86 ± 2.33%, \( n = 8 \) vs. 48.82 ± 3.03%, \( n = 10 \)) and by 50% after reperfusion for 24 h (21.51 ± 1.84%, \( n = 7 \) vs. 43.33 ± 4.67%, \( n = 7 \)), respectively. Administration of GP by intravenous injection 5 min after ischemia (45 min) followed by reperfusion for 4 h reduced infarct size/area at risk by 52% (24.39 ± 2.00%, \( n = 8 \) vs. 51.03 ± 3.69%, \( n = 10 \)) (Fig. 1C and D).

3.2. Glucan phosphate decreased NFκB binding activity

Fig. 2A shows that I/R increased NFκB binding activity in both ischemic (338%) and non-ischemic areas (332%) compared to control groups. GP blunted, but did not completely inhibit, I/R induced NFκB binding activity both in ischemic (47%) and non-ischemic (41%) areas compared to the I/R control (Fig. 2A). Specific binding of NFκB was confirmed by addition of 100-fold excess of unlabeled NFκB or APII oligonucleotides into the EMSA reaction. As shown in Fig. 2B, unlabeled NFκB oligonucleotides competed for the binding proteins, whereas the unrelated AP-II oligonucleotides did not. The predominant protein complex of NFκB containing p50 and p65 subunits in the myocardium was demonstrated by antibody supershift.
assays. Both antibodies shifted the ischemia induced NFκB binding complex (Fig. 2B).

3.3. Glucan phosphate prevented IkBα degradation

Fig. 3 shows that ischemia (15 min/reperfusion 30 min) significantly decreased IkBα protein levels in the cytoplasmic fraction of myocardium in the ischemic (66%) and non-ischemic (41%) areas as compared to the controls. GP treatment attenuated (P < 0.05) I/R induced degradation of cytoplasmic IkBα.

3.4. Glucan phosphate reduced IKKβ and IRAK activity

Fig. 4 shows that I/R increased IKKβ activity both in ischemic and non-ischemic areas. GP treatment blunted the I/R induced IKKβ activity in ischemic (58%) and non-ischemic (66%) areas of the myocardium (Fig. 4). IRAK is a key cascade kinase in the TLR/MyD88 dependent NFκB pathway [2]. Activation of IRAK stimulates NIK activation, which consequently activates IKKs [2]. Therefore, we investigated the effects of GP on IRAK activity in the myocardium following I/R. As shown in Fig. 5, I/R increased IRAK activity both in ischemic (209%) and non-ischemic (272%) areas compared to the controls. Following GP treatment, the IRAK activity also increased 102% in the ischemic area and 122% in the non-ischemic area compared to the controls. Thus GP attenuated the I/R induced increase in IRAK activity by 51% in the ischemic area and by 55% in the non-ischemic area, respectively.

3.5. Glucan phosphate reduced association of TLR4 with MyD88

TLR/NFκB signaling is transmitted through an adapter protein, MyD88, to the downstream kinases, IRAKs. We

Fig. 4. GP attenuated I/R induced IKKβ activity. Rats were treated with GP (40 mg/kg, i.p.) 1 h before the hearts were subjected to ischemia (15 min)/reperfusion (3 h). Immunoprecipitation was performed with IKKβ antibody followed by kinase assay with GST-IκBα as substrate. Tissue samples from the normal and sham controls were taken from the same regions as the ischemic hearts. Results were expressed as means ± S.E.M. of five hearts per group. *P < 0.05 compared to normal control group. #P < 0.05 compared to I/R group. A representative kinase assay showing IKKβ activity is shown at the top of the figure.

Fig. 5. Attenuation of I/R stimulated myocardial IRAK activity by GP. Rats were pretreated with GP (40 mg/kg, i.p.) 1 h before the hearts were subjected to ischemia (15 min)/reperfusion (3 h). Immunoprecipitation was performed with IRAK antibody followed by kinase assay with myelin basic protein (MBP) as substrate. Tissue samples from normal and sham controls were taken from the same regions as the ischemic hearts. Results were expressed as means ± S.E.M. of five hearts per group. *P < 0.05 compared to normal control group. #P < 0.05 compared to I/R group. Representative kinase assay gel showing IRAK activity is presented at the top of the figure.

Fig. 6. GP reduced the association of TLR4 with MyD88. Rats were injected with GP (40 mg/kg) by intraperitoneal injection 1 h before the hearts were subjected to ischemia (15 min)/reperfusion (3 h). Immunoprecipitations were performed with MyD88 antibody followed by immunoblotting with TLR4 specific antibody. Normal (N) and sham (S) samples were taken from the same regions as the ischemic hearts. Results are expressed as means of integrated intensity ± S.E.M. of four hearts per group. *P < 0.05 compared to control. A representative immunoprecipitation followed by immunoblotting gel showing TLR4 and MyD88 protein levels is presented at the top of the figure.

Fig. 7. Glucan phosphate induced PI3K activity in the myocardium following I/R. Glucan phosphate (40 mg/kg) was administered 1 h before the hearts were subjected to ischemia (15 min)/reperfusion (3 h). PI3K activity was analyzed by lipid kinase activity assay. Normal and sham samples were taken from the same regions as in the ischemic hearts. Results are expressed as means of integrated intensity ± S.E.M. of 4–5 hearts per group. *P < 0.05 compared to the controls. A representative lipid kinase assay of PI3K activity is shown in the insert.
investigated whether GP alters the physical interaction of TLR4 with MyD88. As shown in Fig. 6, I/R did not alter the physical interaction of TLR4 with MyD88, but GP treatment reduced the association of TLR4 with MyD88 in the myocardium following I/R.

3.6. Glucan phosphate increased PI3K activity and inhibited cardiac myocyte apoptosis

Since PI3K/Akt activation will prevent cardiac apoptosis [17], we analyzed the effect of GP on PI3K/Akt activity and examined cardiac apoptosis using the TUNEL assay. The levels of PI3K activity were low in control hearts but increased by 56% in I/R hearts (Fig. 7). GP treatment increased PI3K activity by 160%. Fig. 8 shows that I/R increases phospho-Akt levels by 79%. GP treatment increased the levels of phospho-Akt by 163% in I/R hearts, which is consistent with PI3K activity (Fig. 8). Fig. 9 shows that GP inhibited I/R induced cardiac myocyte apoptosis (17.17 ± 1.41% vs. 3.33 ± 0.71%, *P < 0.05, n = 3).

3.7. Glucan phosphate increased TLR4 tyrosine phosphorylation and TLR4 association with p85

Since GP reduced the association of TLR4 with MyD88 and increased PI3K activity, we examined whether GP treatment stimulated TLR4 phosphotyrosine, resulting in increased association with the p85 regulatory subunit of

![Fig. 8. GP increased Akt phosphorylation in the myocardium following I/R.](image1)

![Fig. 9. GP inhibited I/R induced cardiac myocyte apoptosis.](image2)
It is unclear how Akt signaling could serve a protective role. It plays a critical role in stimulating immune and inflammatory responses in myocardial I/R injury. In support of this hypothesis, a number of recent observations strongly imply the TLR/MyD88-dependent signaling in myocardial I/R injury [16]. For example, increased TLR4 mRNA expression has been observed in CHF and in remodeling murine hearts [12], as well as atherosclerotic plaques [13]. Myocardial TLR4 is responsible for increased myocardial inflammatory cytokine production during endotoxin shock [14,15]. Elevated serum LPS in edematous CHF could be responsible for inflammatory cytokine production through TLR4 mediated intracellular signaling [32]. In addition, blocking TLR2 using a specific antibody enhanced oxidative stress-induced cytotoxicity in neonatal rat ventricular myocytes [33].

GP has been shown to modulate innate immunity [25,26]. Administration of GP to trauma and/or surgical patients stimulated conversion from anergy, decreased the incidence of septic complications and improved survival [27]. GP increases long-term survival in septic mice [25], which also exhibited myocardial inflammation and contractile dysfunction. In addition, increased NFκB activity and inflammatory cytokine gene expression have been observed in both septic and myocardial I/R models [7,9,10,30]. Because the protective effect of GP in sepsis positively correlated with blunting tissue NFκB activity [25], we investigated whether GP could induce cardioprotection. Administration of GP 1 h before I/R or 5 min after ischemia significantly reduced infarct size/area at risk. This is a significant observation because GP exerts cardioprotective activity without requiring prolonged pretreatment time. We have previously shown that GP is safe and well tolerated in humans and animals [25,27].

We have reported that I/R increases NFκB activation first in the ischemic area followed by an increase in the non-ischemic area [9], suggesting that in situ I/R induces a global response. GP treatment blunted, but did not completely inhibit, I/R increased NFκB activation in both ischemic and non-ischemic areas. Numerous studies have shown that inhibition of NFκB activation attenuates myocardial I/R injury, with a concomitant improvement in functional recovery, down-regulation of inflammatory cytokines and inhibition of intracellular adhesion molecule-1 (ICAM-1) gene expression [6,10,11]. Taken together, these data suggest that GP may be a therapeutic target for protection of myocardium from I/R injury.

Persistent IKKβ activity during myocardial I/R could be an important molecular mechanism that causes overproduction of immune and inflammatory responses through prolonged NFκB activation [9]. GP treatment inhibited I/R induced myocardial IKKβ activity. This is consistent with our previous report that GP prevented
LPS induced IKKβ activity in a murine macrophage cell line [26]. To investigate how GP modulated I/R induced myocardial IKKβ activity, we analyzed the activity of IRAK, which is an important cascade kinase upstream of IKKβ in the TLR/MyD88-dependent pathway, and examined the association of TLR4 with MyD88 in the myocardium following I/R. GP treatment reduced the association of TLR4 with MyD88 and blunted I/R induced IRAK activity. Reducing the association of TLR4 with MyD88, with subsequent inhibition of IRAK activity, may be pivotal steps in inhibiting TLR/MyD88-dependent NFκB activation in the myocardium following I/R. This concept is supported by a observation that LPS increased the expression of myocardial proinflammatory cytokines and depressed left ventricular (LV) function in wild type mice but not in C3H/HeJ, which are TLR4-deficient [14], suggesting that TLR4 mediated signaling may play an important role in endotoxic and I/R induced proinflammatory response and LV dysfunction.

The PI3K/Akt pathway is an alternative pathway of TLR mediated signaling that does not signal through MyD88 [19]. Stimulation of THP-1 and HEK 293 cells expressing TLR2 and stimulate PI3K/Akt pathway activation [19]. Similar observations have reported for TLR4 [20] and IL-1 receptor activation [21], which shares the TLR intracellular signaling pathway [2]. We and others have reported that GP [34] and zymosan [35], which contains glucan, induce intracellular signaling through TLR2 and stimulate PI3K/Akt pathway activation. Activation of PI3K/Akt promoted cardiomyocyte survival following I/R injury, inhibited I/R induced cardiac myocyte apoptosis [17,18], and limited LPS induced expression of inflammatory mediators [36].

In this study, we observed that GP treatment decreased myocardial injury in response to I/R. The data indicates that GP treatment shifts the TLR mediated activation signal in I/R from a predominantly NFκB pathway to a predominant PI3K/Akt signaling pathway (Fig. 11) and suggests that activation of TLR mediated MyD88 dependent NFκB pathway correlates with myocardial injury following I/R, while activation of the PI3K/Akt signaling correlates with inhibition of cardiac myocyte apoptosis and decreased injury.

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