Inhibition of Toll-like Receptor 4 With Eritoran Attenuates Myocardial Ischemia-Reperfusion Injury

Akira Shimamoto, MD, PhD; Albert J. Chong, MD; Masaki Yada, MD, PhD; Shin Shomura, MD; Hiroo Takayama, MD; Ani J. Fleisig, MD; Matthew L. Agnew, MD; Craig R. Hampton, MD; Christine L. Rothnie, BS; Denise J. Spring, PhD; Timothy H. Pohlman, MD; Hideto Shimpo, MD, PhD; Edward D. Verrier, MD

Methods and Results—C57BL/6 mice received eritoran (5 mg/kg) intravenously 10 minutes before 30 minutes of in situ of transient occlusion of the left anterior descending artery, followed by 120 minutes of reperfusion. Infarct size was measured using triphenyltetrazoliumchloride staining. A c-Jun NH2-terminal kinase (JNK) activation was determined by Western blotting, nuclear factor (NF)-κB activity was detected by gel-shift assay, and cytokine expression was measured by ribonuclease protection assay. Mice treated with eritoran developed significantly smaller infarcts when compared with mice treated with vehicle alone (21.0±6.4% versus 30.9±13.9%; P=0.041). Eritoran pretreatment resulted in a reduction in JNK phosphorylation (eritoran versus vehicle: 3.98±0.81 versus 7.01±2.21-fold increase; P=0.020), less nuclear NF-κB translocation (2.70±0.35 versus 7.75±0.60-fold increase; P=0.00007), and a decrease in cytokine expression (P<0.05).

Conclusions—We conclude that inhibition of TLR4 with eritoran in an in situ murine model significantly reduces MI/R injury and markers of an inflammatory response. (Circulation. 2006;114[suppl I]:I-270–I-274.)

Key Words: ischemia ■ myocardial infarction ■ reperfusion ■ signal transduction

During ischemia-reperfusion (I/R), cell-surface inflammatory receptors are stimulated, leading to activation of signaling mediators, with subsequent transcriptionally regulated production of pro-inflammatory mediators, including cytokines, chemokines, and adhesion molecules, thus effecting inflammation.1 Accordingly, I/R injury may be attenuated through therapeutic targeting of receptors, signaling molecules, transcription factors, or inflammatory mediators.2 Recently, we have focused on a newly characterized receptor called Toll-like receptor 4 (TLR4) and the role that it may play in myocardial I/R (MI/R) injury. TLR4 is a member of the TLR family, all of which bind components of pathogens, thereby participating in the innate immune response.3 TLR4 has been the focus of particular interest since its recognition as the receptor for Gram-negative lipopolysaccharide (LPS), or endotoxin.4 Consequently, most research on TLR4 has focused on its role in sepsis. However, an increasing number of studies suggest that TLR4 may also serve as a proinflammatory receptor in noninfectious disease processes such as I/R.5

We previously reported that the functional mutation of TLR4 in C57/HeJ mice subjected to MI/R injury resulted in an attenuation of myocardial infarction size, a decrease in activation of c-Jun NH2-terminal kinase (JNK) and translocation of nuclear factor (NF)-κB, and a reduction in mRNA expression of inflammatory cytokines (interleukin [IL]-1β, IL-6, and monocye chemotactic protein [MCP]-1).5 To further examine the clinical potential of TLR4 inhibition, we hypothesized that, in accordance with the results of our previous study using TLR4 mutant mice, pharmacological inhibition of TLR4 would also reduce MI/R injury. In the present study, we evaluated the effect of eritoran (α-D-glucopyranose,3-O-decyl-2-deoxy-6-O-[2-deoxy-3-O-[3(R)-3-methoxyxycetyl]-6-O-methyl-2-[[1(11Z)-1-oxo-11-octadecenyl]amino]-4-O-phosphono-β-D-glucopyranosyl]-2-{[1,3-dioxotetradecyl]amino]-1-(dihydrorogen phosphate), tetrasodium salt [Eisai Research Institute of Boston, Inc, Andover, Mass], an antagonist for TLR4,6 to examine the mechanism of TLR4 activation during MI/R injury.

Methods

Animals and Experimental Design

Male C57BL/6 mice aged 7 to 14 weeks weighing 20 to 25 grams (Charles River Laboratory, Inc, Wilmington, Mass) received 50 μL

References


of eritoran (5 mg/kg dissolved in vehicle) or vehicle intravenously 10 minutes before MI/R or sham operation as a control.

A mouse model of in situ regional MI/R was used as previously described. In brief, the mice were intubated and placed under mechanical ventilation after undergoing general anesthesia with pentobarbital sodium (100 mg/kg, intraperitoneally). A left parasternotomy was performed to expose hearts, and a 7-0 silk suture (Softsilk; US Surgical, Norwalk, Conn) was placed around the left anterior descending coronary artery. A snare was placed on the suture, and regional myocardial ischemia was produced by tightening the snare. After 30 minutes of ischemia, the occlusive snare was released to initiate reperfusion up to 120 minutes. Sham-operated control mice underwent the same surgical procedures except that the snare was not tightened.

All animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH), and also with the Guideline of the Animal Care and Use Committee of the University of Washington and the Guideline for Animal Experiments in Mie University Graduate School of Medicine.

Infarct Size
At the completion of reperfusion, the suture was tied and 4% Evans blue dye was injected anterograde via the aortic root to delineate the area-at-risk (AAR). Hearts were then rapidly explanted, rinsed briefly in 0.9% saline, and placed in 1% agarose gel. The heart was sectioned parallel to the atrioventricular canal into ~1-mm sections. Sections were incubated in 1% triphenyltetrazolium chloride for 10 minutes before being placed in formaldehyde. Heart sections were weighed and digitally photographed 24 hours later, and the left ventricle, AAR, and infarct areas were determined by computer planimetry.

Molecular Analysis
Experimental (eritoran or vehicle-treated) and control (sham operated) mice underwent the identical operation described above, except that reperfusion length varied as follows: 5 minutes of reperfusion for mitogen-activated protein kinases (MAPKs) phosphorylation; 60 minutes of reperfusion for NF-κB activity; and 120 minutes for mRNA of cytokines. At the completion of reperfusion, hearts were rapidly explanted and the left ventricle was dissected free, rinsed in 0.9% saline, snap-frozen in liquid N₂, and then stored at −80°C until subsequent analysis.

Western Immunoblotting Assay for Phosphorylation of MAPKs
Whole-cell protein, extracted from frozen tissue samples with ice-cold lysis buffer (Cell Signaling Technology, Inc, Beverly, Mass), were stored at −80°C until the time of assay. Twenty μg of whole-cell protein were loaded onto 15% sodium dodecylsulfate polyacrylamide electrophoretic gels and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with primary antibodies contained in PhosphoPlus p38 MAPK (Thr180/Tyr182), JNK (Thr183/Tyr185), and extracellular signal-regulated kinase (ERK, Thr202/Tyr204) antibody kits (1:1000; Cell Signaling Technology, Inc). Immunoreactivity was quantitated with Immunoblot imaging software and NIH Image 1.62. The ratio of phospho-to-total MAPKs immunoreactivity was determined for each sample, and the results are expressed as fold increase over sham.

Electrophoretic Mobility Shift Assays for Transcription Factor Activities
Myocardial nuclear proteins from frozen tissue samples were isolated as previously described. Each 10-μg of nuclear protein was incubated in a binding reaction with a 32P-end-labeled, double-stranded oligonucleotide containing the human and rodent consensus NF-κB binding, 5′-AGTGGAGGACTTCCACGGC-3′, and activator protein (AP)-1 binding 5′-CGCTTGATGAGTGTCAGCCGGGA-3′ (Promega Co, Madison, Wis), subjected to electrophoresis in native 6% nondenaturing polyacrylamide gels. Densitometry was performed with NIH Image 1.62. The results were expressed as fold activation over sham.

Ribonucleic Acid Protection Assay
Total RNA was isolated from frozen tissue samples by guanidium thiocyanate-phenol-chloroform method. RNA from each tissue was evaluated with RPA III Ribonuclease Protection Assay Kit (Ambion Inc, Austin, Tex) and customized mice cytokines template (Riboquant Multi-Probe Template Set; BD Biosciences Pharmingen, San Diego, Calif) according to the manufacturer’s protocol. Densitometric analysis was performed with NIH Image 1.62. The results were expressed as fold increase over sham after the amount of mRNA for each cytokine normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Statistical Analysis
All data were expressed as mean±SD. The significance of the difference between group means was analyzed by a 2-tailed Student t test after normality confirmed by Kolmogorov-Smirnov test and Shapiro-Wilks test. All statistical analyses were performed with SPSS 11.0 for Mac OS X (SPSS Inc, Chicago, Ill), and a value of P<0.05 was considered to be significant.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Effect of TLR4 Inhibition on Myocardial Infarct Size After I/R
The AAR-to-left ventricle ratio did not differ between vehicle-treated and eritoran-treated groups (eritoran versus vehicle: 42.8±9.0% versus 47.2±6.5%; P=0.216). Pretreatment with eritoran significantly reduced infarct size within the AAR by 32.0% compared with vehicle pretreatment (eritoran versus vehicle: 21.0±6.4% versus 30.9±13.9%; P=0.041; Figure 1).

Effect of TLR4 Inhibition on Activation of Myocardial MAPKs After I/R
Phosphorylation of p38 MAPK was increased in both groups after MI/R compared with sham group (eritoran: 21.0±13.9% versus 6.4±2.6% fold increase, P=0.046 versus sham; vehicle: 5.80±2.6-fold increase, P=0.035 versus sham); there was no significant difference between eritoran and vehicle groups (P=0.468). Phosphorylation of ERK was also increased in both groups after MI/R compared with sham group (eritoran: 8.82±8.5-
fold increase, \( P = 0.007 \) versus sham; vehicle: \( 8.80 \pm 1.39 \)-fold increase, \( P = 0.006 \) versus sham), and there was no difference between those that received eritoran pretreatment or pretreatment with vehicle (\( P = 0.978 \)). MI/R increased phospho-JNK compared with sham (eritoran: \( 3.98 \pm 0.81 \)-fold increase, \( P = 0.004 \) versus sham; vehicle: \( 7.01 \pm 2.21 \)-fold increase, \( P = 0.012 \) versus sham). In contrast to p38 MAPK and ERK activation, eritoran pretreatment reduced the activation of JNK (\( P = 0.020 \) versus vehicle; Figure 2).

**Effect of TLR4 Inhibition on Transcription Factor Activation After I/R**

Compared with the sham group, in vehicle-pretreated animals, there was a significant increase in NF-κB activation, as determined by assay of NF-κB translocation to the nuclear fraction (7.75 ± 0.60-fold increase, \( P = 0.000002 \) versus sham). Pretreatment with eritoran, however, abrogated this activation (2.70 ± 0.35-fold increase, \( P = 0.00007 \) versus vehicle; Figure 3). However, AP-1 was not observed in either group (data were not shown).

**Discussion**

MI/R injury is characterized by a significant inflammatory response.\(^1\) It is unclear how innate immune signaling pathways are initiated during MI/R injury. Recent studies have highlighted the role of the TLR4-mediated signaling pathway in the induction of innate immunity.\(^2\) Although the majority of investigations on TLR4 have focused on its role as the receptor for endotoxin or LPS,\(^4\) it has also been shown to bind multiple endogenous ligands as well.\(^7\) Interestingly, TLR4 is expressed under normal conditions on many tissues not generally thought to have a role in immune function, includ-
The activation of 2 distinct signaling pathways, which are the phosphorylated Inhibitory-κB (IkB) kinase complex (IKKs), consisting of IKKα, IKKβ, and NF-κB essential modulator/IKKγ, and the phosphorylated MAPKs, such as JNK, and thereby induced the activation of transcription factors NF-κB and AP-1, respectively, which translocate into the nucleus, where it promotes the transcription of a wide variety of genes that encode proteins generally involved in the evolution of an inflammatory response or proteins that inhibit apoptotic cell death.10

Eritoran is a second-generation structural analog of the lipid A portion of LPS. In in vivo and in vitro models, eritoran has been shown to be a potent antagonist of the biochemical and physiological effects of LPS by blocking translocation of NF-κB, which results in decreased expression of inflammatory cytokines.6 Eritoran has been shown to be safe in humans11 and is currently undergoing clinical development as a possible therapeutic agent for the treatment of sepsis and for myocardial protection during coronary artery bypass grafting.

It is not clear how TLR4 signaling is initiated during MI/R; some TLR4 ligands induced by oxidative stress during MI/R may bind and activate TLR4 signaling. It is also not clear how eritoran can block TLR4 signaling during MI/R. LPS is one of the chief ligands to TLR4.4 To ensure our mouse model was free from LPS contamination, levels of plasma endotoxin were measured with a chromogenic Limulus amebocyte lysate assay, whose sensitivity is 0.0113 EU/mL, according to the manufacturer's directions (Endotoxin Single Test; Wako Pure Chemical Industries, Ltd, Osaka, Japan). Differences in plasma endotoxin were not detected between the mice with and without operation (0.08 ± 0.06 versus 0.09 ± 0.05 EU/mL; P = 0.90) or between eritoran-treated and untreated mice at the completion of experiment in this series (0.10 ± 0.03 versus 0.09 ± 0.03 EU/mL; P = 0.95). Thus, our results indicate that TLR4 activation during MI/R injury occurs without LPS. However, the LPS antagonist eritoran still retains the capacity to block TLR4 activation in the absence of LPS.

High-molecular-weight of heat shock proteins (HSPs) such as HSP60,12 HSP70,13 and gp96 (the endoplasmic reticulum HSP90)14 have been reported to activate TLR4 and induce proinflammatory cytokines production through MyD88/IRAK/NF-κB signal transduction pathway. However, other reports suggest that HSPs may facilitate the binding of LPS to TLR4, and thereby act as “danger” signals to the immune system, signaling ongoing tissue injury,7 and that TLR4 activation, mediated by HSPs, may be a result of the contaminating LPS and LPS-associated molecules.15

Li et al16 reported that glucan phosphate (GP), which is a 1-3-β-d-linked polymer of glucose isolated from fungal cell wall and a known ligand of TLR4,17 decreased MI/R injury. This suggests that the TLR-mediated activation signal in I/R may shift to a TLR/phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. After GP stimulation, the TLR cytoplasmic domain is phosphorylated by tyrosine kinase and subsequently disassociate from MyD88. Phosphotyrosine of TLR will recruit the PI3K subunit p85, resulting in activation of the PI3K/Akt alternative pathway.18 A major function of activated Akt is to promote cell survival and to inhibit

### Figure 4

**A**  Ribonuclease protection assay of TNF-α, IL-1β, IL-6, MIP-1α, MIP-2, MCP-1, and GAPDH (internal control) mRNA expression. **B**  The vertical axis denotes fold increase over sham after the amount of mRNA for each cytokine was normalized to that of GAPDH mRNA. Values represent mean ± SD of 4 animals in each group. *P < 0.05 vs sham. **P** < 0.05 vs vehicle.

The activation of TLR4 signaling pathway originates from Toll/IL-1 receptor (TIR) domain. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, myeloid differentiation primary response gene 88 (MyD88), was first characterized to play a crucial role. MyD88 possesses the TIR domain in the C-terminal portion, and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLR4. On stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLR4 through interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TNF-associated factor-6 (TRAF6), leading to the expression of proinflammatory cytokines and chemokines, thus potentiating the new protein synthesis of inflammatory mediators.3

### Figure 4

**A**  Ribonuclease protection assay of TNF-α, IL-1β, IL-6, MIP-1α, MIP-2, MCP-1, and GAPDH (internal control) mRNA expression. **B**  The vertical axis denotes fold increase over sham after the amount of mRNA for each cytokine was normalized to that of GAPDH mRNA. Values represent mean ± SD of 4 animals in each group. *P < 0.05 vs sham. **P** < 0.05 vs vehicle.
apoptosis. Activated Akt phosphorylates IKKs and leads to NF-κB activation. In our additional preliminary study, however, eritoran does not activate Akt and can’t inhibit apoptosis (data were not shown).

NF-κB is a key transcription factor in TLR4-mediated MyD88-dependent signaling pathway and plays a critical role in stimulating immune and inflammatory response gene expression. NF-κB activation has been observed in MI/R. AP-1 activation via JNK phosphorylation is another important cascade of TLR4-mediated MyD88-dependent signaling pathway. In our present study, however, AP-1 activation was not seen, although eritoran attenuated JNK, suggesting that activation of the TLR4-mediated NF-κB pathway may be involved in the inflammatory responses to MI/R injury. In support of this hypothesis, a number of recent observations strongly implicate TLR4-mediated signaling in MI/R injury. Numerous studies have shown that inhibition of NF-κB activation attenuates MI/R injury, with a concomitant improvement in functional recovery, downregulation of inflammatory cytokines, chemokines, and adhesion molecule gene expression. Taken together, these data suggest that inhibiting NF-κB by blocking TLR4 may be a therapeutic target for protection of myocardium from I/R injury.

In conclusion, we have shown in a mouse model that the inhibition of the innate immune receptor TLR4 by its antagonist eritoran attenuates the inflammatory response to MI/R, as evidenced by a significant reduction in infarct size decreased NF-κB nuclear translocation, and decreased expression of inflammatory mediators, such as TNF-α, IL-1β, IL-6, MIP-1α, MIP-2, and MCP-1. We therefore corroborate our previous study demonstrating that TLR4 mediates I/R injury in the heart and further validate TLR4’s clinical potential as a therapeutic target to reduce MI/R injury.

Acknowledgments
We thank Dr Seich Kobayashi (Executive Director of Biological Research and Acting Director of Target Identification and Assay Development, Eisai Research Institute of Boston, Inc) for the technical advice of eritoran, as well as Eisai Research Institute of Boston, Inc for their generous gift of eritoran.

Sources of Funding
This work was supported in part by the National Institutes of Health grant 5R01HL061762 and a grant-in-aid for Scientific Research (14370408) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and Bayer Research Fellowship (2003) grant.

Disclosures
None.

References
Inhibition of Toll-like Receptor 4 With Eritoran Attenuates Myocardial Ischemia-Reperfusion Injury


Circulation. 2006;114:I-270-I-274
doi: 10.1161/CIRCULATIONAHA.105.000901

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/114/1_suppl/I-270

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/