Antagonism of Lipopolysaccharide-Induced Blood Pressure Attenuation and Vascular Contractility


Objective—Aim was to assess whether lipopolysaccharide (LPS)-induced decrease of total peripheral resistance depends on Toll-like receptor (TLR)4 signaling and whether it is sensitive to NO-synthase or TLR4 antagonists.

Methods and Results—C3H/HeN mice (control), expressing a functional, and C3H/HeJ mice, expressing a nonfunctional TLR4, were compared. LPS (20 mg/kg) was injected i.p. 6 hours before hemodynamic measurements. L-NAME and SMT, inhibitors of NO production, and Eritoran, a TLR4 antagonist, were tested for their impact on vascular contractility. Aortic rings were incubated for 6 hours with or without LPS (1 μg/mL), or with LPS + Eritoran (2 μg/mL) and their phenylephrine-induced contractility was measured using a myograph. The expression of cytokines in aortic tissue was examined by real-time polymerase chain reaction. In control mice LPS induced a significant decrease of blood pressure and an increase of heart rate, whereas C3H/HeJ remained unaffected. LPS induced an increase of cytokine expression and a depression of vascular contractility only in control mice but not in C3H/HeJ. L-NAME and SMT increased contractility in all rings and restored LPS-dependent depression of contractility. Eritoran prevented LPS-induced loss of contractility.

Conclusions—LPS upregulates cytokine expression via TLR4 and induces attenuation of smooth muscle contractility which can be effectively antagonized. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: ●●●

Sepsis and septic shock are responsible for about 1400 deaths per day on noncardiac intensive care units in the United States.1,2 During bacterial infection virulence factors are released and the immune system reacts by generating inflammatory cytokines3 leading to a lowered total peripheral resistance (TPR) of the circulatory system. This is compensated by an increase in heart rate and cardiac output. In septic shock cardiac output cannot balance the loss in peripheral resistance any longer resulting in a drastic drop in blood pressure and disturbed microcirculation.4

It has been shown that different members of the Toll-like receptor (TLR) family specifically bind different virulence factors from a wide variety of pathogens. Those virulence factors are very important for the pathogenesis of sepsis. Therefore, competitive inhibition of virulence factors at the Toll-like receptor level might be a potential therapeutic option to treat pathogen-induced sepsis and septic shock. LPS is an important virulence factor of Gram-negative bacteria, which binds to and signals via the TLR4/CD14 complex, thereby inducing inflammatory mediators.3,5

Recently it has been demonstrated that a synthetic Lipid-A-analog called Eritoran (E5564, Eisai, currently also used in different clinical trials for the treatment of sepsis) competitively antagonizes LPS at the TLR4/CD14 complex and inhibits the synthesis and induction of sepsis relevant mediators. In addition, Eritoran was able to prevent the negative inotropic effect of LPS on isolated cardiac myocytes.6,7

In addition nitric oxide (NO) and cytokines or adrenomedullin (ADM) appear to be increased during sepsis and play an important role in the regulation of total peripheral resistance (TPR).8–11 It is known that the NO synthase isoenzyme iNOS (inducible NOS or type II NOS) is upregulated in the murine myocardium after LPS stimulation6 and that vascular NO from iNOS is relevant for vasomotor dysfunction during septic shock.12 Early studies on the use of nonselective NOsynthase inhibitors such as L-NAME or L-NMMA have shown that the inhibition of NO can prevent the sepsis associated hypotension, albeit L-NMMA produced a decreased cardiac output.13,14

Therefore selective inhibitors of iNOS may be more suitable to inhibit the loss of TPR and do so without the detrimental effects of total NO inhibition by L-NAME shown in animal15–18 and clinical studies.19

The purpose of this study was to examine the role of TLR4 and inflammatory cytokines, as target genes in the TLR4 signaling cascade, in a murine model of Gram-negative sepsis...
for the regulation of hemodynamic parameters and vascular contractility. The second aim was to elucidate whether TLR4 antagonism or iNOS inhibition may serve as therapeutic targets for the treatment of TPR loss during septic shock.

**Materials and Methods**

**Animal Model**

Twelve- to 14-week-old LPS responsive C3H/HeN (control) mice and LPS hyporesponsive C3H/HeJ mice of both genders were purchased from Charles River (Sulzfeld, Germany). C3H/HeJ mice carry a point mutation in the cytoplasmic region of TLR4, a proline to histidine, leading to the LPS hyporesponsive phenotype.

Mice were housed in pathogen-free cages with free access to water and standard rodent chow. The animals were handled according to the principles of laboratory animal care (NIH publication No. 85-23, revised 1996), and animal procedures were approved by the local committee for animal care.

**In Vivo Stimulation**

Controls and C3H/HeJ mice were injected i.p. with NaCl or LPS (20 mg/kg bodyweight, #L2630, Sigma-Aldrich Chemical) before hemodynamic recordings.

**Hemodynamics**

Baseline hemodynamic parameters were recorded in anesthetized mice (1% isoflurane, flow 1 l/min Forene, Abbott GmbH) under constant regulation of body temperature. A polyethylene catheter (PE 10) was inserted into the right carotid artery. Pressure signals were digitized using a Powerlab (AD Instruments GmbH) and were continuously recorded using Chart for Windows (Version 4.2.3). After a 15-minute stabilization period baseline hemodynamic parameters were recorded for 15 minutes. Afterward the animals were euthanized to allow excision of the aorta thoracica.

**Mulvany Myograph and Contractility Studies**

Periadventitial fat was removed from the explanted abdominal aorta and the lower part was cut into 2-mm rings according to the method of Mulvany and Halpern. Aortic rings were mounted in a Mulvany Myograph (Model 610 mol/L, Danish Myo Technology) in oxygenated Krebs-Ringer Bicarbonate solution (in mmol/L: glucose 5.5, NaCl 118.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25) at a pH of 7.4 and 37°C for 60 minutes. Then resting tension of 10 mN was applied and the rings were allowed to equilibrate for another 45 minutes. Vessel viability was checked by exposure to high potassium solution (125 mmol/L) and 10-6 mol/L phenylephrine (PE, Sigma #P-6126). Afterward concentration-response curves (CRC) were recorded with PE concentrations of 10-5 mol/L to 10-8 mol/L. All agonist solutions were prepared in ascorbic acid stock solution (1 mg/mL) to prevent oxidation of PE. Isometric contractions were digitized using Myodaq V2.01 and converted using Myodata software (Myonic Technology).

**Stimulation With TLR4 Antagonist**

Rings were kept in Dulbeccos Modified Eagle Medium (DMEM) (37°C, 5% CO2, 95% oxygen) as (1) controls or coincubated with either (2) E.coli LPS (1 μg/mL) or (3) LPS (1 μg/mL) and lipid A antagonist Eritoran (2 μg/mL; EISAI; with Eritoran administered every 2 hours). Afterward concentration response curves to PE were recorded as described above. The Eritoran concentration was chosen according to previous in vitro experiments from our group on isolated cardiac myocytes. We tested a concentration of 1 μg/mL Eritoran and 1 μg/mL LPS, which was not able to prevent the cardiac depressive action of LPS. However, 2 μg/mL of Eritoran, exceeding the LPS concentration twice, were sufficient to block LPS associated cardiac depression. Therefore we applied the same concentration of Eritoran in our experiments on aortic rings.

**Inhibition of NOS**

Another set of PE concentration response curves was recorded after 20 minutes incubation of the isolated aortic rings with the nonspecific NOS-inhibitor N-Nitro-l-arginine-methylester (L-NAME, 100 μmol/L, Sigma #N5751) or the specific iNOS inhibitor S-Methylisothiourea (SMT,100 μmol/L, Sigma #67730).

**Cytokine mRNA Isolation and Real-Time PCR**

Animals were injected i.p. with 20 mg/kg BW LPS for 6 hour prior to isolation of the aortae. Aortae were flash frozen in liquid nitrogen (LN2) and conserved at −80°C. For RNA extractions the whole material was homogenized and RNA was isolated as described elsewhere. For initiation of cDNA-synthesis an Oligo-(d)-T15- Primer for Avian Myeloblastosis Virus Reverse Transcriptase was used and 1 μg RNA transcribed to cDNA. To estimate the specific cDNA-amount a PCR for β-actin, as house-keeping gene, was performed. The resulting PCR-products were seperated using 1.5%-agarosegel. Primers for iNos, tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6 and ADM were selected using Primer Express Software (Applied Biosystems, Foster City, CA, USA) (Search parameters: amplificationsize 100 bp, annealing-temperature 60°C, GC-content 60%). The reaction mixtures were prepared using the Master SYBR Green PCR kit (Eurogenetic, Liège, Belgium). Primer sequences and amplicon lengths were defined as shown in the Table. The reaction was processed in a TaqMan PCR system (Applied Biosystems), and the results were analyzed by calculating the ratio of relative target accumulation over relative β-actin accumulation.

**Statistical Analysis**

Numerical results are given as mean±SEM of n observations, where n is the number of tested animals. For analysis of numerical data, Student unpaired t test was used to compare means between groups. Probability values <0.05 (*) are indicated.

**Results**

**Clinical Manifestations of Infection**

Six hours postinjection, clinical manifestations of shock could be observed in LPS-injected controls. These included diarrhea and lethargy beginning 2 hours after injection. C3H/HeJ mice remained unaffected by LPS injection. These
findings are consistent with those reported by numerous groups and confirm the LPS hyporesponsive phenotype of the C3H/HeJ strain and the LPS responsiveness of the control strain.3,5,10

Hemodynamic Results

To demonstrate the fact that LPS induces hemodynamic instability characteristic of septic shock, endotoxin responsive control mice were injected i.p. with *E. coli* LPS (n = 10) or with saline as control (n = 10). In response to a 6-hour challenge of LPS 20 mg/kg bodyweight the mean arterial blood pressure (MAP) originated in a LPS-dependent decrease in both systolic (106.12 ± 2.80 mm Hg in controls and 73.30 ± 5.33 mm Hg in treated mice; Figure 1A) and diastolic blood pressure (80.80 ± 2.3 mm Hg in controls and 58.46 ± 5.26 mm Hg in treated mice Figure 1B). The heart rate of LPS-treated control animals was significantly increased by 120% (612.62 ± 44.87 beats per minute [bpm] versus 512.46 ± 18.38 bpm in control mice; Figure 1C). However, this increase in heart rate was not sufficient to compensate for a loss of blood pressure. In TLR4-deficient mice of the C3H/HeJ strain none of the above mentioned parameters was significantly reduced (Figure 1A–C).

**In Vivo LPS Treatment**

To clarify whether the described decrease in blood pressure is related to changes in vasocontractility we recorded concentration response curves of aortic rings. Six hours after LPS injection mice were euthanized and aortic rings prepared. LPS stimulation led to a significant attenuation of contraction force of aortic rings from control mice at PE concentrations of 10⁻⁷ mol/L to 10⁻⁵ mol/L (Figure 2A) whereas arterial contractility of C3H/HeJ mice remained unaffected (Figure 2B).

**LPS Induced Hypocontractility Through NO**

After treating aortic rings from LPS-injected control mice with the nonspecific NOS-inhibitor, L-NAME in vitro arterial contractility was restored to normal levels at all PE concentrations. Significant differences between L-NAME–treated control rings and L-NAME–treated rings from endotoxinemic animals could not be detected (Figure 2C). In C3H/HeJ mice 10⁻⁷ mol/L to 10⁻⁵ mol/L L-NAME significantly increased contractility compared with contractility of saline-treated animals of the same strain (Figure 2D). The specific iNOS inhibitor SMT was as efficient as L-NAME, leading to complete restoration of the CRC of rings from LPS-treated control animals (Figure 2E).

**Ex Vivo LPS Treatment**

To determine whether the LPS-induced vascular hypocontractility can be antagonised by the TLR4 antagonist Eritoran, we coincubated aortic rings from control mice with LPS or with LPS and Eritoran in DMEM for 6 hours. Afterward, arterial contractility in response to PE was recorded. LPS-dependent attenuation of vascular contractility was lower in ex vivo stimulated rings. This might be attributable to lower LPS concentrations during ex vivo stimulation compared with LPS concentrations used for in vivo stimulation. Coincubation of Eritoran and LPS completely prevented attenuation of arterial contractility in LPS-treated aortic rings from control mice in comparison to rings incubated only with LPS (Figure 2F).

**Expression of Cytokines in the Murine Aorta**

To determine which cytokines are increased in aortic tissue after LPS stimulation we quantified the mRNA amounts of IL-1β, IL-6, TNFα. In addition, iNOS and ADM were also monitored.

LPS application for 6 hours led to a significant increase of mRNA for the proinflammatory cytokines IL-1β, IL-6, TNFα. In addition, iNOS and ADM were also monitored.

LPS application for 6 hours led to a significant increase of mRNA for the proinflammatory cytokines IL-1β, IL-6, TNFα in control mice, but not in LPS hyporesponsive C3H/HeJ mice.

LPS stimulation caused a pronounced induction of iNOS in control mice compared with saline-treated control mice. LPS did not induce a significant upregulation of iNOS in LPS hyporesponsive C3H/HeJ mice. Comparable results were detected for adrenomedullin, showing a significant increase of ADM in LPS-treated control mice but not in LPS hyporesponsive C3H/HeJ mice (Figure 3).
Discussion

The aim of our study was to elucidate whether TLR4 is involved in the regulation of vascular contractility during sepsis. Furthermore, we wanted to clarify whether TLR4 antagonism can serve as therapeutic target during sepsis.

We demonstrate that blood pressure and vascular contractility are attenuated during septic shock. These results are in accordance with previous findings from other groups, proving the functionality of our model. Additionally, our data show that animals lacking a functional TLR4 are not developing LPS-dependent downregulation of circulatory function (Figure 1). A major new finding of this study is that LPS-induced vascular relaxation depends on TLR4 expressed in the vessel wall. Moreover we show for the first time that a vascular block of TLR4 or iNOS can prevent LPS-induced changes of vascular contractility. Thus, Eritoran might serve as a new tool in the clinical treatment of septical hypotension.

LPS stimulation leads to an induction of several target genes of the TLR4 cascade, including different proinflammatory cytokines like IL1, IL6, TNFα, and potent vasodilators such as iNOS and ADM in aortic tissue. Other groups demonstrated that iNOS-deficient mice show an altered response to LPS stimulation, indicating an important role of iNOS for the generation of septic shock. We wanted to elucidate whether direct iNOS inhibition or antagonism of the TLR4 receptor are also able to block LPS-dependent vascular hypocontractility.
Our data show that isolated aortic rings taken from control animals treated with LPS developed a significantly reduced contractility (Figure 2A), which was comparable to rings incubated with LPS in vitro. This demonstrates that circulating immune cells are not necessary for LPS-induced vascular relaxation (Figure 2F). Because there are usually no immune cells like macrophages in the aortic wall, this response has to be induced by local cells, e.g., smooth muscle cells or endothelial cells. Smooth muscle cells are known to express functional Toll Like Receptor 2,30 3,31 and 4.32,33 TLR4 has also been localized on endothelial cells.34,35 Therefore, both cell types are susceptible to TLR4-stimulation and thus LPS is able to induce cytokine as well as iNOS expression in both cell types. The importance of TLR4 is further underlined by the observation that rings from C3H/HeJ animals did not show a loss of contractility after LPS treatment (Figure 2B). The vascular relaxation seems to rely mainly on NO as it can be blocked by L-NAME. SMT was also able to prevent vascular relaxation completely, therefore iNOS appears to play a major role. It has been shown in previous studies that the use of nonspecific NO synthase inhibitors prevents hypotension during sepsis.36–39 In this study we confirmed this effect and show furthermore that arterial iNOS expression is upregulated after LPS treatment (Figure 3), and that nonselective and selective inhibition of iNOS completely restored vascular contractility (Figure 2C-D) in aortae from septic mice.

In addition to the regulation of iNOS we wanted to elucidate how other target genes of the TLR4 cascade are regulated after LPS treatment. We were specifically interested in the regulation of ADM as a potent vasodilator.40 It has been shown that inflammatory stimuli led to an increase of ADM expression and thus to increased angiogenesis and vasodilation.41 Frede et al could show that HIF1α is upregulated in monocytes after LPS stimulation leading to increased ADM expression.42 We were for the first time able to...
demonstrate that ADM upregulation is TLR4-dependent in aortic tissue (Figure 3) and might thus play an important role for catecholamine resistant hypotension in sepsis. However, it remains unclear whether this upregulation of ADM is dependent on the upregulation of HIF1α in aortic tissue or induced by a different pathway.

The LPS-dependent upregulation of ADM should lead to pronounced vasodilatation. There are supposed to be 2 different pathways leading to ADM-dependent vasodilatation: (1) direct increase of cAMP and activation of eNOS, or (2) a combined pathway of cAMP-NO mediated vasorelaxation. Those pathways focus on ADMs influence on NO produced by the NOS isofom eNOS. However our data suggest an additional interaction between ADM and iNOS, because the inhibition of iNOS also completely restored contractility in aortic rings. We therefore assume that in our experimental surrounding ADM-dependent NO is produced mainly by iNOS, not eNOS. The upregulation of iNOS, ADM, and cytokines, after LPS stimulation, was not detected in C3H/HeJ mice (Figure 3).

The most intriguing result of our data are that LPS-induced vascular relaxation can be inhibited by using a competitive inhibitor of TLR4. Eritoran, a second generation analogue of the lipid A component of LPS, prevented the loss of vascular contractility and led to normal vascular contractility during sepsis. However, it is still unclear whether Eritoran prevents cytokine upregulation in vivo and LPS-dependent hypotension in vivo.

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The most intriguing result of our data are that LPS-induced vascular relaxation can be inhibited by using a competitive inhibitor of TLR4. Eritoran, a second generation analogue of the lipid A component of LPS, prevented the loss of vascular contractility and led to normal vascular contractions during phenylephrine administration (Figure 3F). Baumgarten et al have demonstrated that the use of Eritoran is beneficial to maintain cardiac function and myocyte contractility and led to normal vascular contractility during endotoxia. Our data demonstrate that Eritoran is able to protect arterial contractility during sepsis. Thus inhibition of TLR4 by Eritoran might serve as a novel therapeutic concept to prevent cardiac dysfunction and hypotension after endotoxemia. However, further investigations regarding the impact of Eritoran on iNOS regulation are necessary.

Our data provide evidence that LPS-induced attenuation of blood pressure is TLR4-dependent and that the use of selective and nonselective inhibitors of NO synthase can restore vascular contractility during Gram-negative sepsis. Furthermore, the presented data show that the TLR4 is an important target for pharmaceutical modulation of sepsis and that the TLR4 inhibitor Eritoran might improve treatment during sepsis. However, it is still unclear whether Eritoran prevents cytokine upregulation in vivo and LPS-dependent hypotension in vivo.


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