Endotoxin Induces Toll-Like Receptor 4 Expression in Vascular Cells: A Novel Mechanism Involved in Vascular Inflammation

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Introduction

Toll-like receptors (TLRs) are type I transmembrane receptors that expressed on the cell membrane after lipopolysaccharide (LPS) stimulation [1]. TLRs are critical for the induction of downstream signals in atherogenesis during endotoxin-mediated vascular disturbances. Previous studies have demonstrated that TLR4 is abundantly expressed in failing myocardium [2], and in macrophages infiltrating lipid-rich atherosclerotic lesions [3]. Moreover, an association between the functional expression of TLR4 and the subsequent intimal hyperplasia has recently been described [4]. TLR4 mediates and regulates LPS-induced proinflammatory activation in vascular smooth muscle cells (VSMCs) [5]. LPS-induced systemic inflammatory responses, with the resulting proliferation of smooth muscle cells, could increase neointimal formation after balloon injury and stent implantation [6]. TLR4 expression under LPS stimulation is controlled by transcriptional and posttranscriptional mechanisms in mammalian cells [7]. In a series of experiments, we explored the cellular events and the underlying mechanisms involved in LPS-induced TLR4 expression in VSMCs [8,9].

Ginkgo biloba extract (GBE) has been used in traditional Chinese medicine for thousands of years [10]. Both in vitro and in vivo studies have demonstrated the protective effects of GBE on the response to oxidative stress [11]. GBE inhibits the proliferation of cultured VSMCs and decreases the intimal response to balloon injury of the abdominal aorta by reducing IL-1β expression in cholesterol-fed rabbits [12]. GBE has been shown to possess antioxidant and antiatherosclerotic properties. We found that GBE inhibits TNF-alpha-induced reactive oxygen species (ROS) generation, transcription factors activation, and cell adhesion molecules expression in human aortic endothelial cells [13]. In our experiments, we also evaluated the effects of GBE on LPS-induced TLR4 expression in human aortic smooth muscle cells (HASMCs).

LPS Induces TLR4 Expression in HASMCs

LPS significantly induced TLR4 mRNA expression in HASMCs. The expression of TLR4 mRNA was increased and reached a maximum at 2 h after stimulation with LPS. The addition of cycloheximide (a protein synthesis inhibitor) or actinomycin D (a RNA polymerase inhibitor) significantly reduced TLR4 expression in LPS-treated HASMCs, suggesting that the LPS-induced expression of TLR4 requires de novo RNA and protein synthesis, and that short half-life proteins may be involved [14].
TLR4 Expression Is Mediated by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Activation and ROS Generation

LPS or H2O2 significantly induced TLR4 mRNA expression, which was significantly blocked by polyethylene-glycol-conjugated superoxide dismutase (a permeable antioxidant enzyme), N-acetylcysteine (NAC) (a glutathione precursor), apocynin (a specific NADPH oxidase inhibitor), or Rac1 siRNA.

Intracellular Mitogen-Activated Protein Kinase Signaling in TLR4 Expression

Diphenylene iodonium (DPI) and apocynin significantly decreased mitogen-activated protein kinase (MAPKs) (p38 MAPK, ERK1/2, and SAPK/JNK) activation in LPS-stimulated HASMCs, suggesting that NADPH-oxidase-derived ROS are involved in the activation of MAPKs. Real-time polymerase chain reaction (PCR) demonstrated that LPS-induced TLR4 mRNA expression was reduced by SAPK/JNK inhibitor and ERK1/2 inhibitor, but not by p38 MAPK inhibitor suggesting that SAPK/JNK and ERK1/2 play more significant roles than that of p38 MAPK in the transcriptional regulatory signaling pathway. Interestingly, total and membrane TLR4 protein expression was inhibited by SB203580, indicating that p38 MAPK might be involved in the posttranscriptional regulation of TLR4 expression.

Human Antigen R (HuR) Mediates TLR4 mRNA Stability

The t1/2 of TLR4 mRNA deduced for the various conditions indicated that LPS stimulation rapidly increased the stability of TLR4 mRNA in HASMCs. HuR was found predominantly in the nucleus in untreated HASMCs. Treatment with LPS caused a marked accumulation of cytoplasmic HuR over time. LPS-prolonged TLR4 mRNA stability and LPS-induced TLR4 mRNA expression were blocked completely by HuR siRNA suggesting the critical role of HuR in the regulation of TLR4 mRNA expression.

Based on the cytoplasmic localization of HuR in LPS-treated HASMCs and the specific region of AU-rich elements (AREs) recognized by HuR [15], in our studies, we found that treatment with LPS markedly increased the HuR interaction with the 3’ untranslated regions (3’UTR) of TLR4 mRNA in HASMCs.

A reporter plasmid containing the 3’UTR and luciferase reporter gene was transfected into HASMCs to investigate whether the 3’UTR promotes TLR4 mRNA expression. The results showed that treatment with LPS caused a slight increase in luciferase activity compared with unstimulated cells in the cytomegalovirus (CMV)-luciferase plasmid-transfected group and a significant increase in luciferase activity in the CMV-luciferase-TLR4 3’UTR sense plasmid-transfected group. When HASMCs were co-transfected with the HuR RNAi and CMV-Luciferase-TLR4 3’UTR sense plasmid followed by LPS treatment, HuR-specific siRNA effectively blocked the luciferase activity in CMV-Luciferase-TLR4 3’UTR sense plasmid-transfected cells. These findings suggest that the 3’UTR of TLR4 mRNA confers LPS responsiveness and that HuR modulates the 3’UTR-mediated gene expression in HASMCs.

NADPH Oxidase and MAPK-Signaling Pathways Mediate TLR4 mRNA Stability and HuR Expression

An actinomycin D chase experiment was conducted to determine whether LPS affects the
steady-state dynamic balance between the rate of transcription and the message stability of TLR4 mRNA [16]. It was found that pretreated with NAC, DPI, apocynin, or transfected with Rac1 siRNA significantly decreased the LPS-induced extension of the TLR4 mRNA half-life. HASMCs pretreated with DPI or transfected with Rac1 siRNA, also significantly decreased the LPS-induced lengthening of the TLR4 mRNA t_{1/2}. When compared with the control, LPS significantly induced cytoplasmic HuR expression. The addition of DPI or transfection with Rac1 siRNA significantly decreased cytoplasmic HuR expression in HASMCs treated with LPS. A number of RNA-binding proteins that modulate inflammation-related mRNA stability may be regulated by the MAPK pathways [17]. In our studies, we found that LPS-induced lengthening of TLR4 mRNA stability was significantly reduced by p38 inhibitor, and ERK inhibitor, but not by JNK inhibitor. In addition, p38 inhibitor significantly reduced cytoplasmic HuR expression in HASMCs treated with LPS. These results suggest that LPS-induced cytoplasmic HuR expression is mediated by an oxidative stress-related mechanism and the p38 MAPK-signaling pathway in HASMCs.

**Ginkgo biloba Extract (GBE) Can Inhibit the Expression of TLR4 in LPS-induced HASMCs**

In our studies, we demonstrated that GBE inhibits the proliferation in LPS-induced HASMCs [18]. GBE pretreatment also significantly decreased TLR4 mRNA and membrane TLR4 protein expression in HASMCs after LPS treatment. These results suggest that GBE modulates cell proliferation, which is mediated by the suppression of TLR4 in LPS-treated HASMCs. In addition, treatment with GBE appeared to inhibit the translocation of p47^{phox} and activation of Rac1, and to attenuate the phosphorylation of ERK1/2. When the western blot analysis and confocal microscopy were conducted to measure the cytoplasmic level of HuR in HASMCs following treatment with GBE and LPS, it is shown that GBE markedly inhibited the accumulation of cytoplasmic HuR in LPS-stimulated HASMCs. These findings suggest that GBE reduces LPS-induced TLR4 expression through both transcriptional and posttranscriptional regulations.

**Conclusions**

We clearly demonstrated in our studies that LPS-enhanced TLR4 expression and mRNA stabilization in HASMCs is mediated by NADPH-oxidase-related ROS production and MAPK signaling pathways *in vitro* (Figure 1). HuR directly interacts with the 3’UTR of TLR4 mRNA to prolong the stability of TLR4 mRNA in LPS-stimulated HASMCs. Both the Rac1-dependent NADPH oxidase activation and p38 MAPK-signaling pathway play critical roles in LPS-increased HuR activation, which mediate TLR4 mRNA stabilization in HASMCs. Finally, GBE decreased LPS-induced TLR4 expression by inhibiting LPS-induced NADPH oxidase activation, mRNA stabilization, and MAPK signaling pathways (Figure 2). It is suggested that therapy with anti-inflammatory agents is a promising way of preventing vascular diseases.

**References**

2. Frantz S, Kobzik L, Kim YD, et al. Toll4 (TLR4) expression in cardiac myocytes in normal and
Figure 1. The diagram shows that LPS-enhanced TLR4 expression and mRNA stabilization in HASMCs is mediated by NADPH-oxidase-related ROS production and MAPK signaling pathways in vitro.
Figure 2. The diagram shows that *Ginkgo biloba* extract (GBE) inhibited LPS-induced TLR4 expression by inhibiting LPS-induced NADPH oxidase activation, mRNA stabilization, and MAPK signaling pathways.