The Role of Human Antigen R, an RNA-binding Protein, in Mediating the Stabilization of Toll-Like Receptor 4 mRNA Induced by Endotoxin

A Novel Mechanism Involved in Vascular Inflammation

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Objective—Lipopolysaccharide (LPS) interacts with toll-like receptor 4 (TLR4) and induces proliferation of vascular smooth muscle cells (VSMCs) which plays a causal role in atherogenesis. The role of TLR4 expression and regulation in LPS-stimulated VSMCs remains unclear. TLR4 mRNAs often contain AU-rich elements (AREs) in their 3' untranslated regions (3'UTR) which have a high affinity for RNA-binding proteins. It is not known whether the RNA-binding protein, human antigen R (HuR), regulates TLR4 expression in human aortic smooth muscle cells (HASMCs).

Methods and Results—Stimulation of HASMCs with LPS significantly increased the cytosolic HuR level in vitro. Immunoprecipitation and RT-PCR demonstrated that LPS markedly increased the interaction of HuR and 3'UTR of TLR4 mRNA. The reporter plasmid, which contains the 3'UTR of TLR4 mRNA, significantly increased luciferase reporter gene expression in LPS-induced HASMCs. These data suggest that the 3'UTR of TLR4 mRNA confers LPS responsiveness and that HuR modulates 3'UTR-mediated gene expression. Knock-down of HuR inhibited LPS-induced TLR4 mRNA stability in HASMCs and luciferase reporter gene expression in CMV-Luciferase-TLR4 3'UTR-transfected HASMCs. In addition, inhibition of NADPH oxidase activity by diphenylene iodonium, knock-down of Rac1 gene expression by siRNA, and decrease of p38 MAPK activity by SB203580 significantly decreased the cytosolic HuR level, which mediates TLR4 mRNA stability.

Conclusion—Activation of NADPH oxidase and the MAPK-signaling pathway contribute to HuR-mediated stabilization of TLR4 mRNA induced by LPS in HASMCs. In the balloon injured rabbit aorta model, systemic inflammation induced by LPS caused intimal hyperplasia and increased TLR4 and HuR expression. (Arterioscler Thromb Vasc Biol. 2006; 26:2622-2629.)

Key Words: LPS ■ toll-like receptor ■ human antigen R ■ inflammation ■ vascular smooth muscle cell (VSMC)

Epidemiological research suggests that failure of coronary artery bridge grafts or restenosis is brought about by chronic inflammation induced by endotoxin. LPS-induced systemic inflammatory responses could increase neointimal formation after balloon injury and stent implantation, and the resulting proliferation of vascular smooth muscle cells (VSMCs) may play a key role in atherogenesis. Toll-like receptor 4 (TLR4) mediates the cellular activation by LPS. When cells are stimulated by endotoxin, TLR4 leads to activation of p44/p42 mitogen-activated protein kinase (MAPK) and proliferation in VSMCs.

Previous studies have demonstrated that TLR4 is expressed abundantly in failing myocardium and in macrophages infiltrated into human and murine lipid-rich atherosclerotic lesions. The functional expression of TLR4 and subsequent augmentation of intimal hyperplasia have recently been described. Hypoxia diminishes TLR4 expression through reactive oxygen species (ROS) generated by mitochondria. Antecedent resuscitated hemorrhagic shock influences the TLR4 mRNA steady-state level. Although TLR4 expres-

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sion affects cell responsiveness under endotoxin stimulation, and TLR4 expression under LPS stimulation is controlled by transcriptional and posttranscriptional mechanisms,10 TLR4 expression and related mechanisms in VSMCs are still unclear.

The basal expression of proteins associated with inflammatory responses, immunoregulation, oncogenesis, and cell growth is normally very low,11 possibly because of repression of transcription through the potentially unstable expression of mRNAs.12 Unstable mRNAs often contain AU-rich elements (AREs) in their 3’ untranslated region (UTR). The characteristic motif is AUUUA, but the ARE size and AU content may vary. The ARE-mRNA database (http://rc.kfshrc.edu.sa/ared) has clustered ARE into three groups depending on the number of motifs in the ARE stretch; previous reports have demonstrated that ARE-encoded biological diversity results in the occurrence of some diseases.13

ARE-regulated mRNA stability is mediated by RNA-binding proteins, such as human antigen R (HuR), AU-binding factor 1 (AUF 1), and tristetraprolin (TTP).14 HuR is a ubiquitous protein belonging to the embryonic lethal abnormal vision family of RNA-binding proteins, predominantly nuclear proteins, which shuttle between the nucleus and cytoplasm. HuR regulates cyclin A, cyclin B, and p21 mRNA stability during cell proliferation. AUF1, a member of the heteronuclear ribonucleoprotein (hnRNP) family (also known as hnRNP D), is another RNA-binding protein, which exists in four isoforms (37, 40, 42, and 45 kDa). Although AUF1 is thought to destabilize mRNA,17 the AUF1 isoforms have different roles in the regulation of mRNA turnover.18 TTP is critically implicated in inflammation and is a member of zinc finger proteins that bind to AREs and destabilize mRNAs of tumor necrosis factor (TNF-α).19 However, TTP knockout causes a severe inflammatory syndrome in vivo,20 and it is possible that the effects of TTP are countered by HuR.21

Our previous data showed that LPS-enhanced TLR4 expression in human aortic smooth muscle cells (HASMCs) is mediated by mRNA stabilization, although we have not yet clarified whether RNA-binding proteins are involved in this process. The aim of this study was to explore the cellular events involved in, and the mechanisms underlying, the LPS-enhanced TLR4 mRNA stability in HASMCs. We used an animal model to confirm that LPS stimulation rapidly increased the stability of TLR4 mRNA in HASMCs (please see the online data supplement).

Materials and Methods

Cell Culture

The HASMCs and THP-1 cells were used for this study (please see the data supplement, available online at http://atvb.ahajournals.org).

Quantitative Real Time and Traditional Polymerase Chain Reaction

Total RNA was isolated using a TRizol reagent kit (Invitrogen), according to the manufacturer’s instructions. The detailed method was published on http://atvb.ahajournals.org.

Results

LPS Prolongs TLR4 mRNA Stability and Promotes TLR4 mRNA Expression

HASMCs were treated with 25 ng/mL LPS for 2 hours and then actinomycin D for 1 hour. The t1/2 of mRNA deduced for the various conditions indicated that LPS stimulation rapidly increased the stability of TLR4 mRNA in HASMCs (LPS group: 309.2±22.2 minutes versus control group: 58.9±6.9 minutes). Under 25 ng/mL of LPS stimulation, the expression of TLR4 mRNA was elevated at 1 hour (149.5±15.9% of control) and reached maximal level (210.3±13.2% of control) at 2 hours, suggesting that LPS significantly induces TLR4 mRNA expression in HASMCs (please see the online data supplement).
LPS Triggers a Distinct Increase in Cytoplasmic HuR

HuR was found predominantly in the nucleus in nontreated HASMCs. Treatment with 25 ng/mL LPS caused a marked accumulation of cytoplasmic HuR over time (Figure 1A); in contrast, AUF1 expression was found predominantly in the nucleus, and its distribution remained unchanged following LPS treatment (Figure 1B). In Western blot analysis, LPS markedly increased the cytoplasmic level of HuR but not AUF1 (Figure 1C); and the level of nuclear HuR did not decrease concomitantly with the increase in cytoplasmic HuR. The heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 and β-actin were used as internal controls. To identify the TTP expression in HASMCs, traditional RT-PCR and quantitative real-time PCR were used to measure TTP mRNA expression in HASMCs after 100 ng/mL LPS treatment. TTP mRNA was not expressed in quiescent or LPS-stimulated HASMCs; in contrast, as reported previously, the addition of 100 ng/mL LPS rapidly increased the expression of TTP mRNA by THP-1 cells (Figure 1D). These observations suggest that LPS treatment significantly increases cytoplasmic HuR accumulation because of the export of nuclear HuR but does not influences the distribution of AUF1 or expression of TTP.

Knockdown of HuR Gene Inhibits LPS-Induced TLR4 mRNA Expression

Western blot analysis revealed the effective reduction of HuR in the HuR siRNA-transfection group compared with the negative control siRNA-transfection group and the naive control group (Figure 2A). LPS-prolonged TLR4 mRNA stability and LPS-induced TLR4 mRNA expression were blocked completely by HuR siRNA (Figure 2B and 2C); this effect was not observed in the siRNA negative
control, suggesting the critical role of HuR in the regulation of TLR4 mRNA.

**HuR Interacts With the 3′UTR of TLR4 mRNA**

Based on the cytoplasmic localization of HuR in LPS-treated HASMCs and the specific region of ARE recognized by HuR,23 we postulated that the HuR might interact with the 3′UTR of TLR4 mRNA and assessed this possibility using immunoprecipitation and RT-PCR. Protein fractions were subjected to immunoprecipitation with anti-HuR antibody or control pre-immune mouse serum and subjected to polyacrylamide gel electrophoresis. Anti-HuR antibody was efficient in the immunoprecipitation process (Figure 3A). Treatment with LPS markedly increased the HuR interaction with 3′UTR of TLR4 mRNA (Figure 3B). These findings indicate that LPS increases the HuR interaction with 3′UTR of TLR4 mRNA in HASMCs.

The 3′UTR of TLR4 mRNA and HuR siRNA Confer LPS Responsiveness

To investigate whether the 3′UTR promotes TLR4 mRNA expression, a reporter plasmid containing the 3′UTR and luciferase reporter gene were transfected into HASMCs. A schematic representation of the various plasmids containing luciferase and the 3′UTR of TLR4 mRNA are shown in Figure 4A. The CMV-Luciferase plasmid-transfected group had a higher basal luciferase activity than the control groups (naive cells and pcDNA3.1 vector-transfected cells). Treatment with 25 ng/mL LPS caused a slight increase in luciferase activity compared with unstimulated cells in the CMV-Luciferase plasmid-transfected group and a significant increase in luciferase activity in the CMV-Luciferase-TLR4 3′UTR sense plasmid-transfected group (Figure 4B). In contrast, LPS treatment did not change the basal luciferase activity in the CMV-Luciferase-TLR4 3′UTR antisense plasmid-transfected group.

HASMCs were cotransfected with the HuR RNAi and CMV-Luciferase-TLR4 3′UTR sense plasmid followed by LPS treatment. HuR-specific but not the negative control siRNA effectively blocked the luciferase activity in CMV-Luciferase-TLR4 3′UTR sense plasmid-transfected cells stimulated with LPS (Figure 4C). 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 LPS-Induced HuR Expression**

HASMCs were pretreated with 30 μmol/L diphenylene iodonium (DPI) or transfected with 100 μmol/L Rac1 siRNA, which significantly decreased the LPS-induced lengthening of the TLR4 mRNA t½ (LPS group, 349.3 ± 43.2 minutes; DPI-treated group, 122.9 ± 24.3 minutes; Rac1 siRNA-transfected group, 110.2 ± 21.2 minutes) (Figure 5A). Compared with control, LPS significantly induced cytoplasmic HuR expression. Addition of 30 μmol/L DPI or transfection with 100 μmol/L Rac1 siRNA significantly decreased cytoplasmic HuR expression in HASMCs treated with LPS (Figure 5B). Many RNA-binding proteins that modulate inflammation-related mRNA stability may be regulated by the MAPK pathways.14 We examined whether HuR expression in LPS-induced HASMCs is regulated by the MAPK-signaling pathways and found that LPS-induced lengthening of TLR4 mRNA stability was significantly reduced by SB203580, a p38 inhibitor (120.9 ± 12.0 minutes), and PD98059, an extracellular signal regulated kinase (ERK) inhibitor (182.9 ± 15.4 minutes), but not by SP600125, a JNK inhibitor (Figure 5C). In addition, SB23580 significantly reduced cytoplasmic HuR expression in HASMCs treated with LPS (Figure 5D). 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.

**HuR Expression in Endothelia-Denuded Abdominal Aorta of Rabbits With Systemic Inflammation**

To study whether LPS administration affects HuR expression, which is associated with TLR4 expression, immunohistochemical staining was performed using antibodies against TLR4, HuR, and α-actin (to identify smooth muscle cells) on serial sections of abdominal aortas (Figure 6). Compared with sections from the control group, sections showed a slightly thickened intima in the LPS group, markedly thickened intima in the ED group, and severe intimal hyperplasia in the ED+LPS group. Compared with the control group, it also enhances a bit the expression of TLR4 in the ED group; strong TLR4 staining was seen on the luminal surface in the LPS group and in the neointima in the ED+LPS group. Slightly positive HuR staining was seen in the LPS-treated group, and strongly positive HuR staining was seen in the ED+LPS group in the markedly thickened intima. The antibody to α-actin to identify smooth muscle cells showed that TLR4 and HuR were expressed predominantly in smooth muscle cells of neointima.

**Discussion**

Our data revealed that HuR interacts directly with the 3′UTR of TLR4 mRNA to prolong the stability of TLR4 mRNA in LPS-stimulated HASMCs. The Rac1-dependent NADPH oxidase activation and p38 MAPK-signaling
pathway play critical roles in LPS-increased HuR activation, which mediates TLR4 mRNA stabilization in HASMCs. In a balloon injured rabbit aorta model, we also demonstrated that LPS increases the expression of TLR4 and HuR. The data provide evidence for a direct involvement of VSMCs in LPS-mediated inflammatory activation, which may contribute to the progression of cardiovascular disorders.

Figure 5. NADPH oxidase and MAPK signaling pathways-mediated LPS-induced HuR expression regulates TLR4 mRNA stability. A and C, Actinomycin D chase experiments were used to assess TLR4 mRNA stability. B and D, The expression of cytoplasmic HuR was assessed by Western blot analysis. Negative control siRNA (NC siRNA) was used to validate the knockdown. The bar graphs show the relative intensity of each band relative to β-actin as measured by densitometry. Data represent the results from three independent experiments. The bar graphs of B and D show the relative intensity of each band relative to β-actin, which was measured by densitometry. *P < 0.05 compared with the unstimulated group and †P < 0.05 compared with the LPS-treated only group.

Figure 6. Immunohistochemistry to assess TLR4 and HuR expression in rabbit abdominal aorta. The intima was markedly thickened in the ED and ED+LPS groups compared with the control and LPS-treated groups. The black arrowheads indicate smooth muscle cells overlapping with TLR4 (brown signal) and HuR (brown signal) expression. Compared with the control group, it also enhances a bit the expression of TLR4 on the neointima in ED group, stronger TLR4 expression was seen on the luminal surface in the LPS-treated group and on the neointima in the ED+LPS-treated group. HuR expression increased especially in the ED+LPS group compared with the control group. TLR4 and HuR were expressed predominantly in smooth muscle cells, as identified by antibody against α-actin (white arrowheads). Corresponding hematoxylin staining was used for nucleus identification. The lumen is uppermost in all sections, and the internal elastic laminae are indicated by arrows. The scale bar indicates 50 μm.
Controlling the stability of mRNA may modulate gene expression and adjust inflammatory responses efficiently. The stability of mRNA is often modulated by AREs through 3'UTR.11 The characteristic motif of ARE is AUUUA, although the copy numbers and organization of AREs are vary, and there are various classes of ARE.24 We have shown the sequence of 3'UTR of TLR4 mRNA and predicted that the most obvious feature of the nucleotide fragment is the presence of three AU motifs (please see the online data supplement). Our data provide evidence that HuR binds to ARE of TLR4 mRNA and that HuR is an essential regulator of TLR4 expression. In the future, we plan to study the serial deleted constructs to identify the minimal region required for 3'UTR of TLR4 mRNA interaction with HuR; using the site-directed mutagenesis technique to provide evidence of the requirement for the nucleotides in this interaction.

The increased stability of ARE-containing mRNAs has also been linked to an increase in the cytoplasmic level of the endogenous HuR.25 In our LPS-induced HASMCs model, the p38 MAPK signaling pathway is involved in the positive regulation and accumulation of cytoplasmic HuR. Activation of HuR results from the activation of p38 MAPK. MAPKAPK-2, which is phosphorylated by p38 MAPK, regulates the stability of mRNAs for TNF-α,26 through their AREs. Importantly, the activation of MAPKAPK-2, which increases the cytoplasmic accumulation of HuR,27; dominant negative mutants of MAPKAPK-2 changes the cytoplasmic HuR level27 and blocks cytokine-induced COX-2 mRNA degradation. HuR-mediated stabilization of TNF-α mRNA in LPS-induced macrophages results from an increase in HuR methylation.29 Although our data do not clarify the precise mechanisms responsible for p38 MAPK regulation of the increase in cytoplasmic HuR, we speculate that p38 MAPK regulates protein-arginine methyltransferase and increases the methylation of HuR, which would then modulate the interaction of HuR with its ligands, thus affecting its nucleocytoplasmic shuttling. ERK also regulates transportation of cytokine mRNAs from the nucleus to the cytosol in an ARE-dependent manner.30 Our study showed that ERK does not regulate the cytoplasmic HuR level but decreases the stability of TLR4 mRNA, suggesting the involvement of a mechanism independent of cytoplasmic HuR expression.

Intracellular MAPK signaling pathways are associated with vascular inflammation and that this is modulated by ROS.31 In vascular cells, H₂O₂ activates p38 MAPK, JNK/SAPK, and ERK. DPI, NAC, p22phox siRNA, or catalase may inhibit the activation of p38 MAPK and JNK/SAPK-mediated Rac1-dependent H₂O₂ production.32,33 The production of ROS and the activation of the p38 MAPK signaling pathways induce the expression of several redox-sensitive genes associated with atherogenesis. The direct interaction of TLR4 with NADPH oxidase is involved in LPS-mediated ROS generation and NF-κB activation.34 Our previous study found that apocynin and DPI inhibit LPS-induced MAPK phosphorylation in VSMCs (please see the online data supplement). Treatment with DPI and transfection of Rac1 RNAi may decrease the stability of TLR4 mRNA and downregulate the cytoplasmic expression of HuR suggesting that NADPH oxidase-mediated ROS production contributes to the activation of MAPKs, which is associated with the expression of HuR and stability of TLR4 mRNA in redox-sensitivity vascular inflammation.

In summary, LPS-enhanced TLR4 expression and mRNA stabilization in HASMCs is mediated by HuR expression and that this expression is dependent on the NADPH oxidase activation and p38 MAPK signaling pathways in vitro. Using an inflammatory animal model, involving balloon injured vessels, we also found that LPS increase the expression of TLR4 and HuR in neointima hyperplastic lesion in vivo. Our findings suggest that suppressing HuR activation or therapy with anti-inflammatory agents is a promising means of preventing vascular inflammation.

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**Disclosures**

None.

**References**


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Materials and methods

Cell Culture

The HASMCs, (Cascade Biologics, USA) were grown in M231 medium (Cascade Biologics, USA) containing smooth muscle growth supplement and 2% fetal calf serum. HASMCs under passage 8 were used for this study. Human monocytic cell line THP-1 (American Type Culture Collection, VA, USA) were cultured in the RPMI 1640 medium (Gibco Inc., USA) containing 10% fetal calf serum and maintained in suspension at 0.5-1.0 x 10^6 cells/mL.

Quantitative Real Time and Traditional Polymerase Chain Reaction

Total RNA was isolated using TRIZOL reagent kit (Invitrogen, CA, USA). cDNA was synthesized from total RNA using Superscript® II reverse transcriptase. Quantitative real time polymerase chain reaction (PCR) was performed using a FastStart DNA Master SYBR Green I kit and LightCycler (Roche, CA, USA). FastStart Taq DNA polymerase was activated by incubation at 95 °C for 2 min, before 40 cycles of 95 °C for 1 s, 60 °C for 5 s, and 72 °C for 7 s. Fluorescence was measured at 86 °C after the 72 °C extension step. The level of TLR4 and TTP mRNA expression were determined in arbitrary units by comparison with an external DNA standard that were amplified by the TLR4 and TTP primers, respectively. Traditional PCR was performed using Promega PCR reagents. Amplification of GAPDH was performed in the same samples to verify RNA abundance. The PCR mixture was amplified in DNA thermal cycler (Biometra T3, Berlin, Germany) with 30 cycles for GAPDH (denaturation at 95°C for 1 min, annealing at 53°C for 1 min and extension at 75°C for 2 min) and 35 cycles for TTP (denaturation at 95°C for 1 min, annealing
at 55 °C for 1 min and extension at 75 °C for 1 min). PCR primers used for amplification of TLR4, TTP and GAPDH were: TLR4 forward primer: 5’-AAG CCG AAA GGT GAT TGT TG-3’, reverse primer: 5’-CTG TCC TCC CAC TCC AGG TA-3’; TTP forward primer: 5’-CGC CCA CTC TCT GAG AAG GTC3’, reverse primer: 5’- TTC GCC CAC TCG AAC CTC-3’, GAPDH forward primer: 5’-TGC CCC CTC TGC TGA TGC C-3’, reverse primer: 5’-CCT CCG ACG CCT GCT TCA CCA C-3’. All specific primers were synthesized by Sigma-Aldrich (MO, USA).

**Actinomycin D Chase Experiments**

Actinomycin D (20 μg/mL) was added to cells for 1 h following the treatments under various experimental conditions. Total RNA was extracted at 0, 15, 30, 60, 120, 180, and 240 min after the addition of actinomycin D, and quantitative real time PCRs was then performed. mRNA decay curves were constructed, and the half-life (t1/2) was calculated from the curves.

**Confocal Microscopy**

Cells were plated on cover slips, grown to confluence, and then treated with 25 ng/mL LPS as indicated in the figure legends. After the treatment, the cells were fixed with 4% formaldehyde-PBS for 15 min. Cell membranes were fenestrated with 0.4% Triton-100-PBS, and nonspecific binding sites were blocked with 2% BSA-PBS-Tween 20 (0.1% v/v). The cells were incubated with rabbit anti-HuR (Chemicon, CA, USA) or rabbit anti-AUF1 antibody (Upstate, VA, USA) and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC). The slides were observed with confocal microscopy.

**Western Blot Analysis**

Cytoplasmic and nuclear fractions were prepared and modified as previously, and Western blot analysis was conducted to determine the levels of HuR and AUF1 in
HASMCs. Protein was subjected to SDS-PAGE and electrophoretically transferred to a PVDF membrane; the membrane was probed with rabbit anti-HuR or rabbit anti-AUF1 antibody and then incubated with horseradish peroxidase-conjugated secondary antibody and developed using the enzyme-linked chemiluminescence detection reagents. Mouse anti-β-actin (Labvision/NeoMarkers, CA, USA) and mouse anti-hnRNP C1/C2 (Abcam, Cambridge, UK) antibodies were used as loading controls.

**Knockdown Gene Expression with Interference RNA**

Knockdown HuR gene expression by transfection was measured with small interfering RNA (siRNA), in which 10⁶ cells were trypsinized and resuspended in 100 μl of Nucleofector solution (Amaxa, Germany), and 100 μM of siRNA duplexes (Ambion, USA) were electroporated as advised in the instruction manual. The HuR siRNA (Ambion Catalog #16704) were as follows: GGA UGA GUU ACG AAG CCU GTT and CAG GCU UCG UAA CUC AUC CTG; Silencer Validated siRNA (negative control siRNA, Ambion Catalog #4635) was used to validate the knockdown. Cells were seeded onto six well plates immediately posttransfection, and for further experiments at 48 h later after transfection.

**Cross-linking Immunoprecipitation Assay of RNA-protein Interaction**

To determine whether HuR interacts directly with the 3’UTR of TLR4 mRNA, immunoprecipitation and RT-PCR were carried out with modifications based on a previous report². To induce cross linking, cells were irradiated directly with ultraviolet-B light for three times with 4000 mJ on ice cold PBS. Cells were lysed with cold cell lysis buffer, and RNA-protein complexes were extracted with centrifugation. For immunoprecipitation, 500 μg of cytoplasmic fraction aliquots were incubated with 10 μg of antibody that recognizes HuR and protein G-sepharose. The
RNA in the immunoprecipitated material was used in quantitative real time PCR reactions to detect the presence of 3’UTR of TLR4 mRNA. The mRNA was reverse-transcribed using a Reverse-iT™ 1st Strand Synthesis Kit (AB gene, USA), followed by quantitative real time PCR to measure the 3’UTR transcripts levels. PCR primers were designed for the 3’UTR of TLR4 mRNA as GAA CTG GGT GTT CAC TTT TTC C and ATC CCA GCC ATC TGT GTC TC.

**Luciferase Reporter Assay and Transfection**

The 10^6 cells were trypsinized and resuspended in 100 µl of Nucleofector solution; 1 µg of the reporter plasmid (CMV-Luciferase-TLR4 3’UTR sense, and CMV-Luciferase-TLR4 3’UTR antisense) was transfected using the Nucleofector electroporation device according to the manufacturer's instructions. Equal amounts of the pcDNA3.1 vector (mock plasmid) or luciferase reporter gene contained pcDNA3.1 plasmid (CMV-Luciferase) were used as control groups. Uniform transfection efficiencies were normalized using a β-galactosidase reporter plasmid. Cell extracts were prepared with reporter lysis buffer (300 µl/well), protein concentrations were determined, and the luciferase activity was quantified by luminometry (Wallac Victor^2, Finland) using the luciferase assay system (Promega, USA). β-galactosidase activity was measured using a β-galactosidase enzyme assay kit (Stratagene, USA).

**Animal Balloon-injury Experiment**

All animals were treated under protocols approved by the Institutional Animal Care Committee of the National Yang-Ming University (Taipei, Taiwan, ROC). The experimental procedures and animals’ care conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Twenty-three adult male New Zealand White
rabbits (2.5 to 3 kg) were provided 60 g/kg/day of commercial normal chow diet and water ad libitum. Balloon injury of abdominal artery was performed as described previously.³ Briefly, a 3F Fogarty embolectomy catheter (Biosensor, USA) was inserted through the femoral artery of anesthetized rabbits and passed to the abdominal aorta (16 cm), inflated with normal saline, and withdrawn four times. Heparin (100 unit/kg) was administered immediately after arteriotomy. Arterial blood was collected from the ear artery into sodium citrate-containing tubes. The animals were sacrificed after five weeks, and the vessels were collected for immunohistochemical staining.

**Immunohistochemical Staining**

Immunohistochemical staining was performed on serial 5 µm thick paraffin-embedded sections from rabbit abdominal aortas using anti-TLR4, anti-HuR and anti-α-actin antibodies. For negative control staining, non-specific IgG was substituted for HuR-specific and TLR4-specific antibodies.

**Discussion**

LPS is considered a strong stimulator of atherosclerosis.⁴ TLR4 is expressed in vascular cells and is critical for the induction of downstream signals in atherogenesis in LPS-mediated vascular disturbances. The LPS-induced systemic inflammatory responses could increase neointima formation after balloon injury and stent implantation so that the resulting proliferation of VSMC plays a key role in atherogenesis.⁵ TLR4 in VSMCs may play a major contributory role in vascular inflammation and cardiovascular disorders.⁶ LPS treatment could upregulate TLR4 expression and promote a pro-inflammatory phenotype in VSMCs, which may play an active role in vascular inflammation.⁶
A number of RNA-binding proteins have high affinity for AREs, which control mRNA stability in inflammation. AUf1, a prototypic RNA-binding protein, is generally regarded as a destabilizer of mRNA. In contrast, overexpression of AUf1 isoforms has been implicated in the differential regulation of cytoplasmic mRNA turnover. TTP is strongly implicated in the regulation of inflammation-related mRNAs. However, TTP binds to ARE of TNF-α mRNA, and suppresses TNF-α promoter activity, which results in mRNAs deadenylation and degradation.10 HuR binds to ARE-containing mRNAs of GM-CSF, c-FOS, and TNF-α,11 and slows the decay of mRNAs in vitro.12 HuR stabilizes inflammation-related mRNAs, suggesting that HuR is a critical posttranscriptional modulator of inflammation. Although numerous studies have indicated that cytoplasmic HuR can stabilize specific mRNAs, contrasting reports have also shown that inducible increases in HuR in murine innate compartments suppress inflammatory response in vivo. Studies have also show that HuR synergizes with the T cell-restricted intracellular antigen, a translational silencer, to reduce the translation of cytokine mRNAs in vitro.13 HuR appears to have pleiotropic roles in inflammation, and the functions and mechanisms of HuR remain to be elucidated.

HuR is not the only trans-acting factor regulating mRNA stability by cis-regulatory elements; AUf1 competes with HuR in target mRNAs and regulates mRNA stability by endonucleases.14 Downregulation of HuR results in AUf1-mediated degradation of sGCα1 mRNA and sGCβ1 mRNA in genetic hypertension, suggesting that the ratio of HuR and AUf1 activity regulates vascular function.15 The role of HuR during the response to the signaling of cell proliferation has been described in previous paper.16 Our data showing that HuR interacts with 3’UTR of TLR4 mRNA in LPS-stimulated HASMCs and regulates the target mRNA
stability suggest that HuR is a contributing factor to VSMC mediated vascular inflammation. Less expression of cytoplasmic and nuclear HuR in VSMCs has been found in normal coronary and renal arteries than in samples from atherosclerotic lesions and sclerotic arterialized vein grafts.\textsuperscript{17} We used a rabbit abdominal aorta denudation model with LPS administration to investigate whether LPS augmented the expression of TLR4 and HuR in arteries after vessel injury because recent findings have illustrated a repertoire of TLR4 associated with atherosclerotic lesions and characterized by the upregulated expression of HuR in VSMCs in lesions. In contrast, we detected AUF1 in LPS-induced HASMCs using Western blot analysis and confocal microscopy, and in LPS-stimulated aorta injured rabbits using immunohistochemistry; we also found that indistinct expression of AUF1 in HASMCs and in atherosclerotic lesion suggesting that AUF1 may have different roles in different cell types and tissues.\textsuperscript{18}
Figure I: Regulation of TLR4 mRNA expression in HASMCs by LPS treatment. A, HASMCs were stimulated with 25 ng/mL LPS for 0–4 h. TLR4 mRNA expression was analyzed by quantitative real-time PCR after normalization to GAPDH mRNA. B, HASMCs were pretreated with 25 ng/mL LPS for 1 h before actinomycin D treatment for 1 h. TLR4 mRNA stability was evaluated by Actinomycin D chase experiment. Transcription was blocked by actinomycin D treatment at t = 0. Total RNA was extracted at various time points and quantitative real-time PCR was performed.
Figure II: The 3’UTR of human TLR4 mRNA. The oligonucleotides for the PCR synthesis of 3’UTR of TLR4 mRNA are marked by arrows and AU motifs are predicted and marked by underlining.
Figure III: To clarify whether NADPH-oxidase-derived ROS are involved in the activation of MAPK signaling pathways, we treated HASMCs with 30 μM DPI or 100 μM apocynin (APO) for 1 h before they were treated with 25 ng/mL LPS for 15 min. DPI and apocynin significantly decreased MAPK (p38 MAPK, ERK1/2, and SAPK/JNK) activation in LPS-stimulated HASMCs, suggesting that NADPH-oxidase-derived ROS are involved in the activation of MAPK signaling pathways.
References


