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Ginkgo biloba Extract Inhibits Endotoxin-Induced Human Aortic Smooth Muscle Cell Proliferation via Suppression of Toll-like Receptor 4 Expression and NADPH Oxidase Activation

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Toll-like receptor 4 (TLR4) initiates the inflammatory response in blood vessels in reaction to immune stimuli such as lipopolysaccharide (LPS) produced by Gram-negative bacteria. LPS-induced proliferation and functional perturbation in vascular smooth muscle cells play important roles during atherogenesis. *Ginkgo biloba* extract is an antiatherothrombotic Chinese herbal medicine with anti-inflammatory properties. The effects of *G. biloba* extract on LPS-induced proliferation and TLR4 expression and the underlying mechanisms for these actions, in human aortic smooth muscle cells (HASMCs), were examined in vitro. LPS-induced proliferation was mediated by the expression of TLR4 in HASMCs. LPS increased the expression of TLR4 in HASMCs, and this effect was mediated by the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, phosphorylation of intracellular mitogen-activated protein kinases (MAPKs), and increases in the cytoplasmic level of HuR and TLR4 mRNA stability. *G. biloba* extract inhibited LPS-induced HASMC proliferation and decreased the expression of TLR4 by inhibiting LPS-induced NADPH oxidase activation, mRNA stabilization, and MAPK signaling pathways. These results suggest that LPS-induced TLR4 expression contributes to HASMC proliferation and that *G. biloba* inhibits LPS-stimulated proliferation of HASMCs by decreasing TLR4 expression.

KEYWORDS: ***Ginkgo biloba*; toll-like receptor 4; proliferation; inflammation; vascular smooth muscle cells**

INTRODUCTION

Toll-like receptors (TLRs) expressed on the cell membrane can be induced by lipopolysaccharide (LPS) stimulation (*1*). First defined in *Drosophila*, TLRs are type I transmembrane receptors that have leucine-rich repeats in the extracellular domain and conserved homology to the mammalian interleukin-1 (IL-1) receptor in the cytoplasmic domain (*2*). TLR4 is critical for the induction of downstream signals in inflammation during endotoxin-mediated vascular disturbance. Epidemiological studies suggest that endotoxins, such as LPS, increase the risk for cardiovascular disorders (*3*). Failure of coronary artery bridge grafts or restenosis of postpercutaneous transluminal coronary angioplasty can be caused by constitutive chronic infection or inflammation (*4*).

During inflammatory processes, TLR4 recruits MyD88 to the intracellular domain of the receptor, leading to the activation of signaling pathways and the production of chemokines, which play important roles in vascular diseases. Previous studies have demonstrated that TLR4 is expressed abundantly in failing myocardium (*5*) and in macrophages infiltrating lipid-rich atherosclerotic lesions (*6*). An association between the functional expression of TLR4 and the subsequent augmentation of intimal hyperplasia has been described recently (*7*). Although endothelial cells are the first vascular cells that come into contact

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with circulating LPS, vascular smooth muscle cells (VSMCs) may also contact circulating LPS, and this interaction may cause the loss of endothelial integrity of the vascular system. TLR4 mediates the expression of cyclooxygenase-2, tumor necrosis factor α (TNF-α), IL-1β, and chemokine in response to exposure to Gram-negative bacteria (*8*). It also regulates LPS-induced proinflammatory activation in VSMCs (*9*). LPS-induced systemic inflammatory responses that result in VSMC proliferation and a possible increase in neointimal formation after balloon injury and stent implantation may play key roles in atherogenesis (*10*). TLR4 exists in and may contribute significantly to the crucial pathophysiological relationship between inflammation and cardiovascular disorders (*11*).

Ginkgo biloba extract has been used in traditional Chinese medicine for thousands of years (*12*). *G. biloba* extract is extracted from *G. biloba* leaves, which contain a defined complex mixture of 24% ginkgo flavone glycoside and 6% terpen lactones (ginkgolides and bilobalides). Both in vitro and in vivo studies have demonstrated the protective effects of *G. biloba* extract on the response to oxidative stress (*13*). In endothelial cells, *G. biloba* extract directly scavenges superoxide anions, hydroxyl radicals, and peroxyl radical species (*14*) and decreases lipid peroxidation, LDH release, and the expression of adhesion molecules (*15*). *G. biloba* extract increases intracellular GSH concentration and GSSG reductase activity (*16*). *G. biloba* extract 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 (*17*).

Our previous studies showed that LPS induces TLR4 expression in VSMCs by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitogen-activated protein kinase (MAPK) signaling pathways, and mRNA stabilization (*18*). Although previous studies have shown that *G. biloba* extract possesses antioxidant and antiatherosclerotic properties, it is unknown whether it directly inhibits LPS-induced TLR4 expression and prevents subsequent LPS-induced cell proliferation in human aortic smooth muscle cells (HASMCs). In this study, we examined the mechanisms of LPS-induced HASMC proliferation and evaluated the actions of *G. biloba* extract on LPS-induced proliferation and TLR4 expression in HASMCs. Results indicate that LPS induces HASMC proliferation and that this effect is mediated by the expression of TLR4. The LPS-induced increase in TLR4 expression in HASMCs is mediated by the activation of NADPH oxidase, the phosphorylation of intracellular MAPKs, and increases in TLR4 mRNA stability. Our data indicate that *G. biloba* extract can inhibit the proliferation and decrease the expression of TLR4 in LPS-induced HASMCs.

MATERIALS AND METHODS

Reagent. The 100 mL *G. biloba* extract stock solution (4% in purified water) contained 960 mg of flavone glycoside and 240 mg of terpen lactone (Dr. Willmar Schwabe, Karlsruhe Inc., Germany).

Cell Culture. HASMCs (Cascade Biologics, Portland, OR) were grown and passaged as described previously (*18*, *19*). Before treatment or stimulation with reagents, the cells were serum-starved for 24 h.

BrdU Incorporation Assay. HASMCs were seeded on 96-well plates at a density of 1×10^4 cells/well. Cells were incubated with various concentrations of *G. biloba* extract for 24 h and then stimulated with 25 ng/mL of LPS for 8 h. DNA synthesis was measured using a 5-bromo-20-deoxyuridine (BrdU) Cell Proliferation Kit (Calbiochem, Darmstadt, Germany). Briefly, after 4 h of incubation with LPS, BrdU labeling solution (BrdU concentration = 10 μM) was added to the cells and incubated for another 4 h. In other studies, 10 μg/mL of functional grade purified mouse anti-TLR4 antibody (eBioscience, San Diego, CA) or IgG2a isotype control antibody (eBioscience) was used before treatment with LPS and BrdU. After removal of the culture medium, the cells were fixed and nucleases were added to partially digest cellular DNA. Anti-BrdU antibody was then added before the addition of the mouse IgG−peroxidase conjugate. The signal was developed with tetramethylbenzidine solution in darkness. Absorbance in each well was measured using a spectrophotometric plate reader at 450 nm with a reference wavelength at 595 nm.

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated using a TRIzol reagent kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized from 2.5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time Polymerase Chain Reaction (PCR) was performed using a LightCycler (Roche, Nutley, NJ) and a FastStart DNA Master SYBR Green I kit (Roche). 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 mRNA expression was determined in arbitrary units by comparison with an external DNA standard, which was amplified with the TLR4 primers. PCR primers used for the amplification of TLR4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were as follows: TLR4 forward primer, 5′-AAG CCG AAA GGT GAT TGT TG-3′; TLR4 reverse primer, 5′-CTG TCC TCC CAC TCC AGG TA-3′; GAPDH forward primer, 5′-TGC CCC CTC TGC TGA TGC C-3′; GAPDH reverse primer, 5′-CCT CCG ACG CCT GCT TCA CCA C-3′.

Extraction of Cellular Protein. Membrane fractions were prepared as described previously (*20*), with modifications. Briefly, HASMCs were lysed in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, and 0.5 μg/mL leupeptin, pH 7.5). Cell lysates were centrifuged. The pellets were resuspended in the lysis buffer and designated the membrane fraction. Total cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF, pH 7.5). The protein concentration was determined with a Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA), and the samples were stored at −70 °C.

Western Blot Analysis. Western blot analysis was used to determine the changes in cell-surface levels of TLR4, NADPH oxidase component $p47^{phox}$, the cytosolic activation of p38 MAPK, extracellular regulated kinase (ERK 1/2), and c-Jun N-terminal kinase (SAPK/JNK) as well as cytoplasmic human antigen R (HuR) level in HASMCs stimulated with LPS. Proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly(vinylidene fluoride) (PVDF) membrane, and then the membranes were blocked with 5% milk. The PVDF membranes were probed with goat anti-TLR4 (1:100; R&D Systems, Minneapolis, MN), mouse anti-$p47^{phox}$ (1:500; BD Biosciences, San Jose, CA), rabbit anti-SAPK/JNK, rabbit anti-phospho-SAPK/JNK, rabbit anti-p44/p42 MAPK, mouse anti-phospho-p44/p42 MAPK (1:1000; Cell Signaling Technology, Danvers, MA), or mouse anti-HuR (1:1000; Upstate, Charlottesville, VA) antibody. Then PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). Mouse anti-α-actin (Labvision/NeoMarkers, Fremont, CA) and rabbit anti-Gαs (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used as loading controls. Protein expression levels were quantified as optical densities, using ImageQuant software v. 5.2.

NADPH Oxidase Activity Assay. NADPH oxidase activity was determined with superoxide-dependent lucigenin chemiluminescence, as previously described (*18*).

Pull-down Assay for Rac1 Activity. Rac1 activation was measured using a glutathione *S*-transferase-(p21-activated kinase)-p21 binding domain (GST-[PAK]-PBD) fusion protein, which binds activated Rac1, as has been described previously (*18*).

Actinomycin D Chase Experiments. Actinomycin D (20 μg/mL for 1 h) was added to HASMCs following treatments under various experimental conditions. Total RNA was extracted at 0, 15, 30, 60,

120, 180, or 240 min after the addition of actinomycin D, and quantitative real-time PCR was performed. mRNA decay curves were constructed, and half-lives ($t_{1/2}$ values) were calculated according to the following formula: $t_{1/2} = 0.693/\kappa$, where $\kappa = \ln(N_0/N_t)/t$, where N_0 represents the cross-point of real-time PCR at $t = 0$ and N_t represents the cross-point at time t.

Confocal Microscopy. Cells were plated on cover slips, grown to confluence, and then treated with 25 ng/mL LPS. 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 in PBS−Tween 20 (0.1% v/v). The cells were incubated with mouse anti-HuR antibody and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC). The slides were observed with confocal microscopy.

Statistical Analyses. Values were expressed as means ± SEM. Statistical evaluation was performed using Student's *t* test and one- or two-way ANOVA followed by Dunnett's test. A P value of <0.05 was considered to be significant.

RESULTS

TLR4 Mediates Proliferation in LPS-Induced HASMCs. To study whether LPS induces proliferation in HASMCs and whether TLR4 mediates the phenomenon, we used a BrdU incorporation assay and TLR4 antibody competition assay. LPS significantly induced BrdU incorporation in a time-dependent manner, as shown in **Figure 1A**. The addition of mouse anti-hTLR4 antibody before LPS treatment significantly reduced the BrdU incorporation in HASMCs (**Figure 1B**). As a negative control in the competition assay, nonspecific IgG2a isotype antibody was substituted for TLR4-specific antibody and did not affect the LPS-induced proliferation of HASMCs. These results suggest that LPS induces the proliferation of HASMCs and that this effect is mediated by TLR4. Treatment of control cells with 5% fetal bovine serum (FBS) for 8 h resulted in a 3.2 ± 0.5-fold increase in BrdU incorporation above baseline (**Figure 1A**). However, cells pretreated with specific-TLR4 antibody did not block the FBS-induced proliferation of HASMCs (**Figure 1B**), suggesting that FBS-induced proliferation of HASMCs is not mediated by TLR4. We then examined whether LPS stimulation of HASMCs leads to a delayed increase in the expression of TLR4 mRNA after the initial lower expression of TLR4 on the cell membrane. Pretreatment of HASMCs with anti-hTLR4 antibody significantly inhibited LPS-induced TLR4 mRNA expression (**Figure 1C**), suggesting that LPS induces TLR4 mRNA expression in HASMCs by TLR4 expressed originally on the HASMC membrane.

***G. biloba* Extract Inhibits Cell Proliferation and TLR4 Expression in LPS-Treated HASMCs.** To examine whether *G. biloba* extract inhibits the LPS-induced proliferation of HASMCs, the BrdU cell proliferation assay was used. Pretreatment of HASMCs with 50 and 100 ng/mL of *G. biloba* extract for 24 h before LPS treatment significantly inhibited LPS-induced proliferation (**Figure 2A**), suggesting that *G. biloba* extract inhibits the proliferation in LPS-induced HASMCs, which is mediated by the suppression of TLR4. We next investigated whether *G. biloba* extract affects TLR4 expression in LPS-induced HASMCs. The TLR4 mRNA and protein were analyzed by quantitative real-time PCR and western blot analysis, respectively. HASMCs were pretreated for 24 h with 25−100 μg/mL of *G. biloba* extract and exposed to 25 ng/mL of LPS stimulation for 2 h. Total RNA was then extracted from the HASMCs. **Figure 2B** shows that LPS induced significant TLR4 mRNA expression. The expression of induced TLR4 mRNA was 203.5 ± 14.3% of the control value after 2 h of LPS stimulation. Pretreatment with 50 or 100 μg/mL of *G. biloba* extract for 24 h significantly inhibited LPS-stimulated TLR4 mRNA expression. Similar to the expression of mRNA,

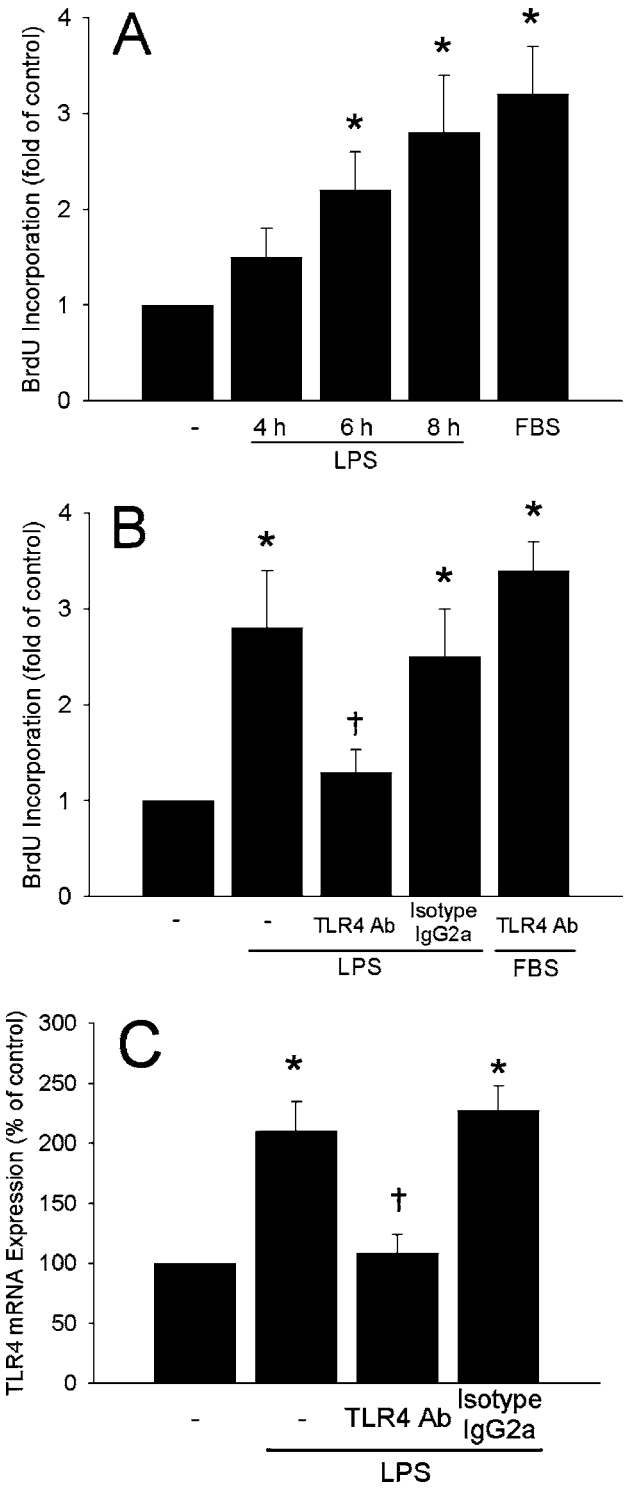

Figure 1. TLR4 mediates cell proliferation in LPS-induced HASMCs. (**A**) HASMCs were treated with 25 ng/mL LPS or 5% FBS for 4 h. The proliferation of HASMCs was examined by BrdU incorporation assay. (**B**) HASMCs were treated with 10 μg/mL of mouse anti-TLR4 or IgG2a isotype antibody before being treated with 25 ng/mL LPS or FBS. BrdU incorporation assay was performed for cell proliferation. (**C**) TLR4 mRNA expression was analyzed by quantitative real-time PCR after normalization to GAPDH. All experiments were performed in triplicate wells; data represent the results of three independent experiments (means ± SEM; *, $P < 0.05$, compared with unstimulated group; †, $P < 0.05$, compared with the LPS-treated group).

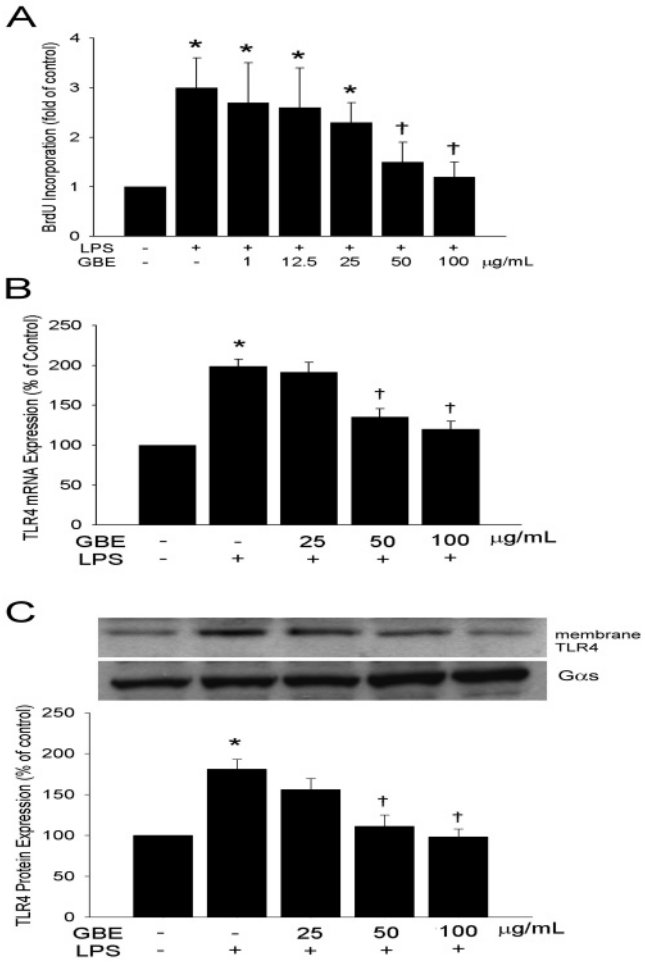

Figure 2. *G. biloba* extract inhibits cell proliferation and TLR4 expression in LPS-treated HASMCs. (**A**) Proliferation of HASMCs was analyzed by BrdU incorporation assay. (**B**) TLR4 mRNA expression was analyzed by quantitative real-time PCR after normalization to GAPDH. (**C**) Membrane TLR4 protein expression was analyzed by western blot. Bar graphs in western blot data show relative intensity of each band (relative to Gαs), which was measured by densitometry. Data represent results from three independent experiments (mean ± SEM; *, $P < 0.05$, compared with unstimulated group; †, $P < 0.05$, compared with LPS-treated group).

G. biloba extract pretreatment significantly decreased membrane TLR4 protein expression after LPS treatment for 4 h (**Figure 2C**). These results suggest that *G. biloba* extract modulates TLR4 expression in LPS-treated HASMCs.

Inhibition of LPS-Induced TLR4 Expression in *G. biloba* Extract Is Mediated by Decreased NADPH Oxidase Activity. To explore whether NADPH oxidase-mediated ROS are involved in LPS-induced TLR4 expression, before LPS stimulation, HASMCs were pretreated with inhibitors of NADPH oxidase, diphenylene iodonium (DPI), and apocynin. LPS or H_2O_2 significantly induced TLR4 mRNA expression (**Figure 3A**). This effect was blocked significantly by 30 μM DPI or 100 μM apocynin, suggesting that LPS-induced TLR4 expression is related to NADPH-oxidase-mediated ROS production. Pretreatment of HASMCs with 25−100 μg/mL of *G. biloba* extract almost completely inhibited the LPS-induced activation of NADPH oxidase (**Figure 3B**). To examine further whether the inhibitory effect of *G. biloba* extract on NADPH oxidase was associated with the translocation of p47^{phox} and activation of Rac1, membrane p47^{phox} and activated Rac1 were analyzed by western blotting and GST-(PAK)-PBD fusion protein pull-down assays, respectively. HASMCs were pretreated with *G.*

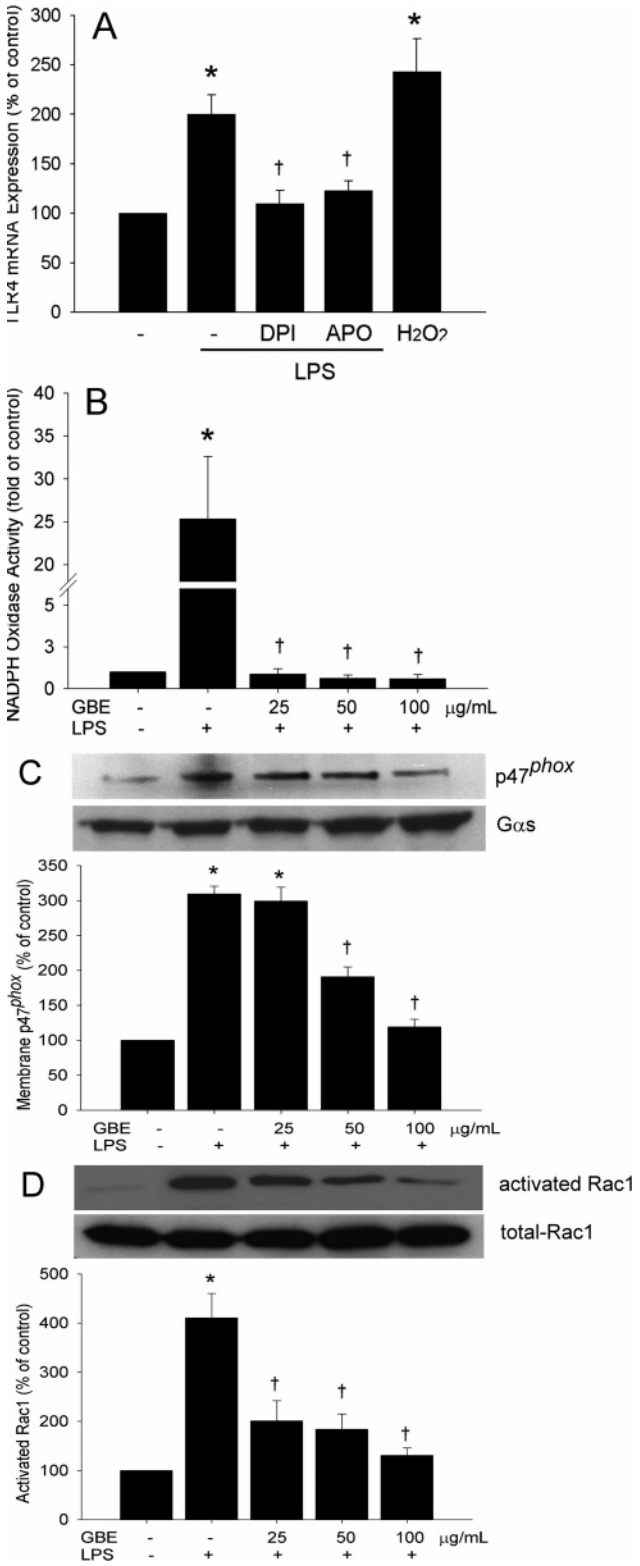

Figure 3. *G. biloba* extract inhibits LPS-induced TLR4 expression mediated by decreasing NADPH oxidase activity. (**A**) TLR4 mRNA expression levels were analyzed by quantitative real-time PCR. H_2O_2 (100 μM) was used as the positive control. (**B**) NADPH oxidase activity was measured with a superoxide-dependent lucigenin chemiluminescent assay. (**C**, **D**) Membrane p47^{phox} levels and cytosolic Rac1 activity were measured by western blotting and pull-down assay, respectively. Bar graphs show the relative intensity of each band (relative to that of Gαs or total-Rac1), which was measured by densitometry. Data represent the results of three independent experiments (means ± SEM; *, $P < 0.05$, compared with the unstimulated group; †, $P < 0.05$, compared with the LPS-treated group).

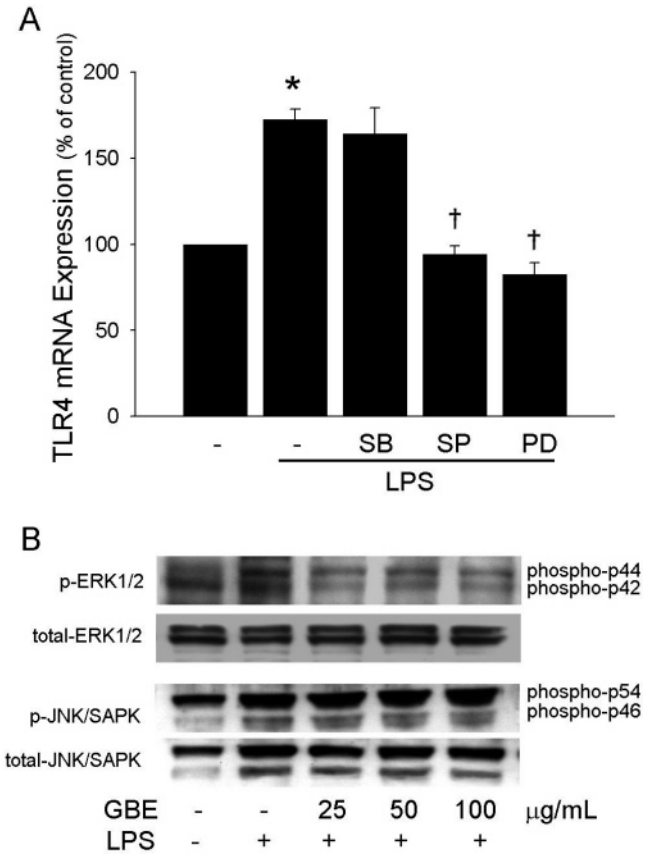

Figure 4. *G. biloba* extract reduces ERK1/2 activation, which mediates LPS-induced TLR4 expression. (**A**) TLR4 mRNA was evaluated by quantitative real-time PCR. Data represent the results of three independent experiments (means ± SEM; *, $P < 0.05$, compared with unstimulated group; †, $P < 0.05$, compared with the LPS-treated group). (**B**) Phosphorylation of ERK1/2 and SAPK/JNK was analyzed by western blotting.

biloba extract and then treated with LPS for 60 min. LPS treatment rapidly induced $p47^{phox}$ translocation to the cell membrane [(5.6 ± 0.3)-fold increase relative to the control value] and the activation of Rac1 [(5.4 ± 0.4)-fold of the control value]. Treatment with *G. biloba* extract appeared to inhibit the translocation of $p47^{phox}$ (**Figure 3C**) and activation of Rac1 (**Figure 3D**). These results suggest that *G. biloba* extract inhibits LPS-induced TLR4 expression and that this effect is mediated by decreased NADPH oxidase activity.

***G. biloba* Extract Reduces LPS-Induced TLR4 Expression through Transcriptional Regulation.** To determine the role of MAPKs in LPS-induced TLR4 expression, we pretreated HASMCs with MAPK inhibitors before LPS stimulation. The real-time PCR demonstrated that LPS-induced TLR4 mRNA expression was reduced by SP600125 (a stress-activated protein kinase, SAPK/JNK, inhibitor) and PD98059 (an extracellular signal-regulated kinase, ERK1/2, inhibitor), but not by SB203580 (a p38 MAPK inhibitor) (**Figure 4A**). This suggests that SAPK/JNK and ERK1/2 play more significant roles than p38 MAPK in the transcriptional regulatory signaling pathway of LPS-mediated TLR4 mRNA expression. The western blot analysis (**Figure 4B**) demonstrated that the phosphorylation of ERK1/2 increased after exposure to LPS, reaching (2.9 ± 0.5)-fold of the control value. *G. biloba* extract pretreatment attenuated the phosphorylation of ERK1/2, but not that of SAPK/JNK.

***G. biloba* Extract Reduces LPS-Induced TLR4 Expression by Posttranscriptional Regulation.** TLR4 expression under LPS stimulation is controlled by transcriptional and posttranscriptional mechanisms (*21*). To determine whether *G. biloba* extract affects the TLR4 mRNA steady dynamic balance between the rate of transcription and message stability, an actinomycin D chase experiment was conducted. The $t_{1/2}$ values deduced for the various conditions according to a formula indicated that LPS stimulation induced rapid changes in the stability of TLR4 mRNA in HASMCs (299.5 ± 34.2 min in LPS treated vs 59.3 ± 7.1 min in the control condition). *G. biloba* extract pretreatment decreased the LPS-enhanced stability of TLR4 mRNA and shortened its half-life (270.6 ± 20.31 min for 25 μg/mL, 160.6 ± 17.6 for 50 μg/mL min, and 24.3 ± 2.5 min for 100 μg/mL of *G. biloba* extract; **Figure 5A**).

HuR is a ubiquitous RNA-binding protein, predominantly a nuclear protein, which shuttles between the nucleus and cytoplasm. HuR stabilizes p21 mRNA (*22*) during cell proliferation. Our previous studies have shown that LPS induces an increase in the cytoplasmic level of HuR and that HuR interacts with TLR4 mRNA directly, which prolongs the half-life of TLR4 mRNA in HASMCs (*23*). We investigated whether *G. biloba* extract pretreatment affects the subcellular distribution of HuR in LPS-induced HASMCs. Under confocal microscopy, HuR was found predominantly in the nucleus in naive HASMCs. LPS treatment caused a marked accumulation of cytoplasmic HuR over time (20 and 120 min) (**Figure 5B**). Pretreatment of *G. biloba* extract significantly decreased the cytoplasmic level of HuR in LPS-treated HASMCs. To confirm the results of confocal microscopy, western blot was conducted to measure the cytoplasmic level of HuR in HASMCs following treatment with *G. biloba* extract and LPS. *G. biloba* extract markedly inhibited the accumulation of cytoplasmic HuR in LPS-stimulated HASMCs (**Figure 5C**). These results suggest that *G. biloba* extract reduces LPS-induced TLR4 expression through posttranscriptional regulation and that this effect is mediated by cytoplasmic HuR expression.

DISCUSSION

In our in vitro model, we found that LPS induced TLR4 mRNA expression in HASMCs and that TLR4 mediated the LPS-induced proliferation of HASMCs. The up-regulated expression of TLR4 by LPS suggests that LPS promotes a change to the proinflammatory phenotype in VSMCs, which may play an active role in vascular inflammation. The up-regulation of TLR4 was mediated by activation of NADPH oxidase, SAPK/JNK, or ERK1/2 signaling. In addition, LPS enhanced the stabilization of TLR4 mRNA transcripts. The traditional Chinese herbal medicine, *G. biloba* extract, may inhibit the LPS-induced proliferation of HASMCs, which is mediated by TLR4 suppression. In LPS-treated HASMCs, *G. biloba* extract may inhibit the expression of TLR4 by decreasing NADPH oxidase activity, inhibiting ERK1/2 signaling, suppressing cytoplasmic HuR level, or shortening the TLR4 mRNA half-life. These data provide evidence that *G. biloba* extract has potential as a therapeutic agent in the treatment of cardiovascular disorders.

At the molecular level, signaling in response to proatherogenic agents both requires and causes the generation of ROS (*24*). NADPH oxidase enzymes represent major sources of ROS (*25*) and are important determinants of the redox states in vascular cells (*26*). The activation of NADPH oxidase is associated with vascular diseases in humans (*27*), and activation of this enzyme mediates the NF-κB activation and proliferation of VSMCs (*28*). NADPH oxidase activity is required for endothelial cell

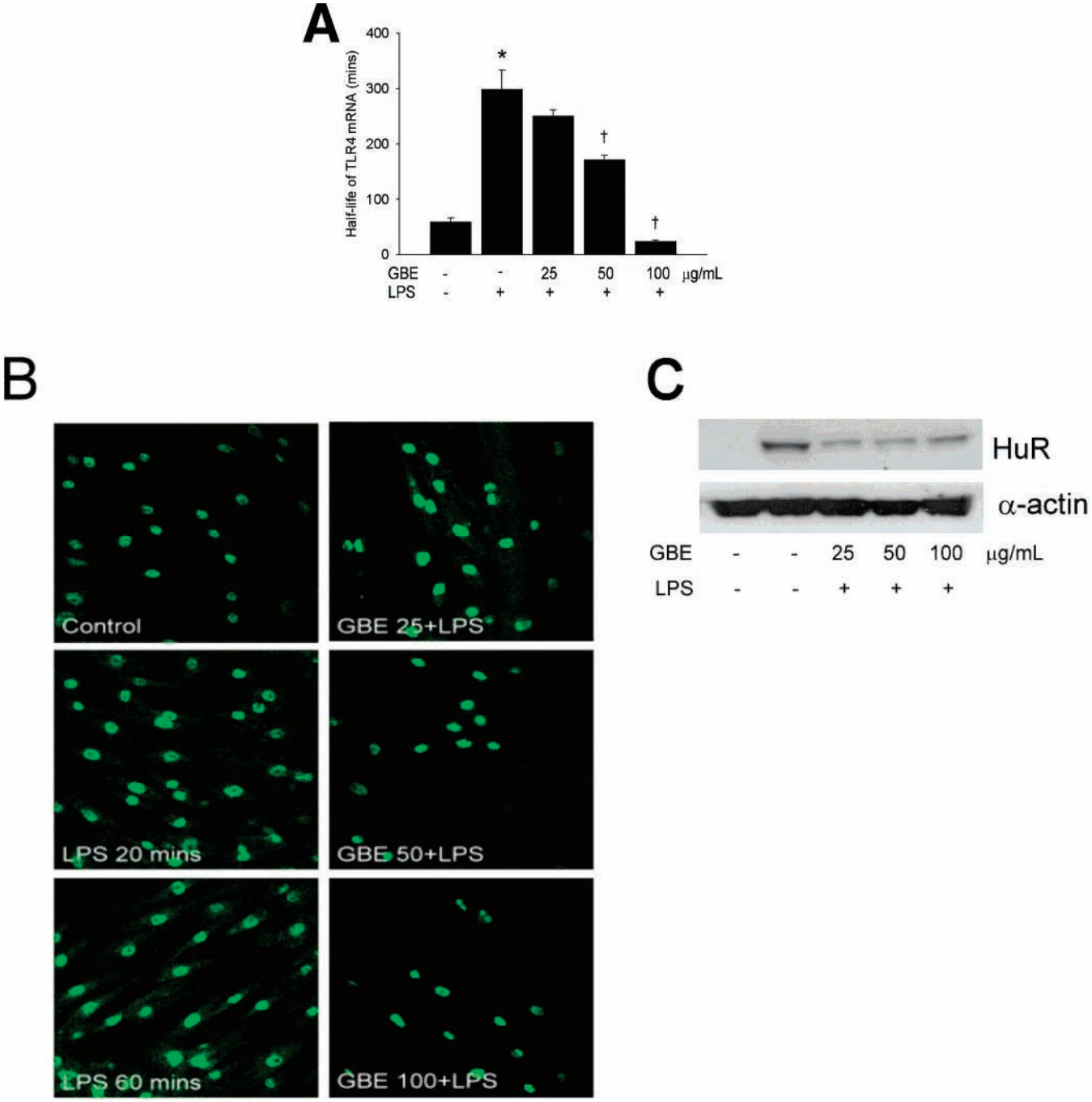

Figure 5. *G. biloba* extract inhibits LPS-induced TLR4 mRNA stability prolongation mediated by inhibition of cytoplasmic HuR level. (**A**) Actinomycin D chase experiment was performed to evaluate TLR4 mRNA stability. The half-life of TLR4 mRNA was calculated according to the following formula: $t_{1/2} = 0.693/\kappa$, where $\kappa = \ln(N_0/N_t)/t$, where N_0 represents the cross-point of real-time PCR at $t = 0$ and N_t represents the cross-point at time t. Data represent the results of three independent experiments (means ± SEM; *, $P < 0.05$, compared with unstimulated group; †, $P < 0.05$, compared with the LPS-treated group). (**B**) Distribution of HuR was identified with immunocytofluorescence and observed by confocal microscopy. (**C**) Cytoplasmic distribution of HuR in HASMCs was detected by western blot analysis. β-Actin was used as internal control.

proliferation and migration (*29*). LPS-induced proinflammatory responses such as NF-κB activation and the subsequent expression of chemokines and adhesion molecules can be controlled by NADPH oxidase 4 in human HEK293, U937, and endothelial cells (*29*).

Changes in TLR4 expression directly affect the cellular responsiveness to LPS. The up-regulation of TLR4 increases the host's sensitivity to antigen and induces host defense responses (*30*). Many factors may cause the increase in TLR4 expression in cells. TLR4 expression increases in HUVECs exposed to shear stress (*31*). Antecedent resuscitated hemorrhagic shock influences TLR4 mRNA steady-state levels (*21*). Previous papers have suggested that NOX interacts directly with TLR4 and that this interaction is essential for the LPS-induced production of ROS and NF-κB, as well as for AP-1 activation (*32*). Although it is unknown how the components of activated NADPH oxidase interact directly with TLR4, these data suggest the existence of a proatherosclerotic vicious cycle of increased NADPH oxidase-dependent superoxide anion formation, TLR4 expression, and further potentiation of oxidative stress by LPS.

Because ROS from NADPH oxidase are involved in the progression of vascular inflammation, it is not surprising that antioxidant therapy is one of the most effective and promising strategies against atherogenesis. Many drugs that are protective against vascular inflammation exhibit anti-NADPH oxidase effects, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and peroxisome proliferator-activated receptor α (PPARα) activators. This leads to suppression of the activity of NADPH oxidase and decreased production of most potent free radical superoxide anions. In addition to the action of statins and PPARα activators, our data show that ROS produced from Rac1- and $p47^{phox}$-mediated NADPH oxidase activation induce TLR4 expression in HASMCs. *G. biloba* extract may decrease the LPS-stimulated NADPH oxidase activation, supporting the notion that *G. biloba* extract has potential applications in the treatment of clinical vascular diseases mediated by NADPH oxidase.

G. biloba extract increases the production of Cu−Zn superoxide dismutase mRNA and up-regulates heat shock protein 70 transcripts in response to stress. Regarding the antiatherogenic effects, recent studies have shown that *G. biloba* extract inhibits TNF-α-induced generation of ROS and the transcription factors NF-*κ*B and AP-1, activates cell adhesion molecule expression in endothelial cells (*15*), inhibits oxidized low-density lipoprotein-stimulated proliferation in VSMCs (*17*), and reduces LPS-interferon-*γ*-induced expression of the inducible nitric oxide synthase gene by inhibiting p38 in macrophages (*33*). *G. biloba* extract also significantly attenuates intimal thickening in the balloon-injured abdominal aorta of cholesterol-fed rabbits by suppressing IL-1*β* expression and inhibiting VSMC proliferation (*17*). All of these effects have implications for atherosclerotic disease.

G. biloba extract is available as a standardized preparation (commercial name, Cerenin). It is the most widely sold phytomedicine in Europe, where it is used to treat the symptoms of early-stage Alzheimer's disease, vascular dementia, peripheral claudication, and tinnitus of vascular origin. It is also one of the 10 best-selling herbal medications in the United States, where *G. biloba* extract is classified as a dietary supplement. The mechanism of action of *G. biloba* is believed to involve its functions as a neuroprotective agent, antioxidant, free radical scavenger, membrane stabilizer, and inhibitor of platelet-activating factor (*15*, *33*, *34*). Other pharmacologic effects include endothelial relaxation mediated by inhibition of 3′,5′-cyclic guanosine monophosphate phosphodiesterase (*12*), inhibition of age-related loss of muscarinergic cholinoceptors and α-adrenoceptors, and stimulation of choline uptake in the hippocampus. *G. biloba* also inhibits *β*-amyloid deposition (*35*). However, the effects of the major components of *G. biloba* extract, flavone glycoside and terpen lactones, on atherosclerosis and vascular inflammation remain to be investigated.

In summary, we found that LPS-enhanced TLR4 expression in HASMCs is mediated by NADPH oxidase activation, mRNA stabilization, and ERK1/2 signaling. This induction is blocked by *G. biloba* extract, suggesting that its anti-inflammatory ability is responsible for some of the beneficial effects of *G. biloba* extract. The amelioration of infection-associated endotoxemia and a therapy directed against the vascular effects of endotoxin using anti-inflammatory agents, such as *G. biloba* extract, may be a promising means of preventing vascular inflammatory disturbances.

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