

Histidine-rich glycoprotein ameliorates endothelial barrier dysfunction through regulation of NF- κ B and MAPK signal pathway

Short title: HRG's regulation of vascular endothelial cells in vitro and in vivo

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ABSTRACT

BACKGROUND AND PURPOSE: Microvascular barrier breakdown is a hallmark of sepsis which associated with sepsis-induced multi-organ failure. Histidine-rich glycoprotein (HRG) is a 75-kDa plasma protein that was demonstrated to improve the survival of septic mice through regulation of cell shape, spontaneous ROS production in neutrophils and adhesion of neutrophils to vascular endothelial cells. We investigated HRG's role in the LPS/TNF- α -induced barrier dysfunction of endothelial cells in vitro and in vivo and the possible mechanism, to clarify the definitive roles of HRG in sepsis.

EXPERIMENTAL APPROACH: EA.hy 926 endothelial cells were pretreated with HRG or human serum albumin before stimulation with LPS/TNF- α . A variety of biochemical assays were applied to explore the underlying molecular mechanisms how HRG protected the barrier function of vascular endothelium.

KEY RESULTS: Immunostaining results show HRG maintains the endothelial monolayer integrity by inhibiting cytoskeleton reorganization, losses of VE-cadherin and β -catenin, focal adhesion kinase degradation and cell detachment induced by LPS/TNF- α . HRG also inhibited the cytokine secretion from endothelial cells induced by LPS/TNF- α , which was associated with reduced NF- κ B activation. Moreover, HRG effectively prevented the LPS/TNF- α -induced increase in capillary permeability in vitro and in vivo. Finally, western blot results demonstrated that HRG prevented the phosphorylation of mitogen-activated protein kinase family (MAPK) and RhoA activation, which are involved mainly in the regulation of cytoskeleton reorganization and barrier permeability.

CONCLUSIONS: Taken together, our results demonstrate that HRG has protective effects on vascular barrier function in vitro and in vivo which may be due to the inhibition of MAPK family and Rho activation.

Key Words: endothelial cells, barrier dysfunction, histidine-rich glycoprotein, sepsis

Abbreviations:

HRG	histidine-rich glycoprotein
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cells
DMEM	Dulbecco' s modified Eagle medium
LPS	lipopolysaccharide
TNF- α	tumor necrosis factor-alpha
FAK	focal adhesion kinase
MAPK	mitogen-activated protein kinase
p-MLC	phosphorylated-myosin light chain
ROS	Reactive Oxygen Species

Bullet point summary**What is already known ?**

HRG has an anti-septic effect in mice through the maintenance of neutrophil quiescence.

What this study adds ?

HRG produced significant protective effects on vascular endothelial cells in vitro and in vivo.

Clinical significance:

The rapid decrease in plasma HRG in sepsis contributes to dysregulation of vascular endothelial cells.

Introduction

Sepsis is a leading cause of death with infection worldwide (Riedemann et al., 2003). It is a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016; Vincent et al., 2013). Septic organ dysfunction is due mainly to an overwhelmed systemic inflammatory process, characterized by the activation of inflammatory cells as well as the enhanced production and release of various cytokines (Gill et al., 2014). Endothelial dysfunction is one of key processes involved in the pathogenesis of sepsis, and it is reported to be associated with patient mortality in clinic (Backer et al., 2002; Trzeciak et al., 2007). It is generally agreed that endothelial dysfunction promotes the neutrophil adhesion to vascular endothelial cells, coagulation abnormalities, microvascular leakage, and hypoperfusion leading to sepsis-induced multiorgan failure (Aird, 2003). Thus, the recovery and maintenance of the body's endothelial barrier function must be critical to survival in cases of sepsis. These processes are likely to provide novel targets for the treatment of sepsis.

The endothelial barrier is maintained mostly by the balance between its intrinsic contractility and intercellular adhesive interactions (Hoelzle et al., 2011; Huveneers et al., 2012). The intrinsic contractility — which is produced by the cytoskeleton rearrangement, the cortical actin dissolution, and stress fiber formation — can result in endothelial retraction and gap formation (Dejana et al., 1995; Jean et al., 2014; Nwariaku et al., 2003). The actin cytoskeleton of endothelial cells is tethered to adherent focal adhesions (FAs), which mediate cell-matrix interactions (Wu, 2005; Burridge et al., 1988). Cell-cell contacts are mediated by adherent junctions among endothelial cells, and these contacts are composed mainly of VE-cadherin. It is a endothelium-specific transmembrane adhesion molecule that regulates the cytoskeleton reorganization via an interaction with actin filaments through catenin binding, which also plays critical roles in modulating endothelial permeability (Gumbiner, 1996; Rodrigues and Granger, 2015; Luscinskas et al., 2001).

Hyperpermeability is a hallmark of different inflammatory diseases such as ARDS and sepsis and aberrant actin dynamics during sepsis leads to intercellular junction destabilization, vascular

hyperpermeability and immune cell recruitment. Therefore, the actomyosin contractility machinery has become the focus of many studies investigating sepsis. (Schnoor et al., 2017). In this process, Rho A triggers the formation of stress fibers through the phosphorylation of myosin light chain ([MLC](#)) by Rho-associated kinase ([ROCK](#)) (García Ponce et al., 2016). Phosphorylated MLC (p-MLC) enables the myosin molecule to change conformation, interact with actin, and slide along its filaments, leading to increased contractility. Members of the mitogen-activated protein kinase family (MAPK) family also interact with the cytoskeleton and in particular, the activated [ERK1/2](#) or [p38](#) was shown to mediate changes in actin dynamics, leading to stress fiber formation, redistribution of VE-cadherin junctions, and permeability changes in endothelial cells (Nwariaku et al., 2002; Huveneers et al., 1997).

The activation of vascular endothelium in sepsis conditions results in the production of various proinflammatory molecules including leukocyte adhesion molecules as well as soluble cytokines and chemokines (Pober and Cotran, 1990). The activation of NF- κ B in endothelial cells is required for the release of proinflammatory cytokines, including interleukin-1 β ([IL-1 \$\beta\$](#)), [IL-6](#), and [TNF- \$\alpha\$](#) (Baeuerle and Baltimore, 1996). Therefore, the inhibition of NF- κ B activation will control the over-production of cytokines in sepsis conditions, which further protects the barrier function in the pathogenesis of sepsis.

Histidine-rich glycoprotein (HRG) is a 75-kDa plasma protein. Human HRG is synthesized in the liver and presents in blood at the concentration range of 60–100 μ g/ml (Koide et al., 1986). The primary structure of human HRG is predicted to be a 507-amino acid multidomain polypeptide consisting of two cystatin-like regions at the N-terminus, a histidine-rich region (HRR) flanked by proline-rich regions (PRR), and a C-terminal domain. HRG is an adaptor protein that interacts with many ligands, including Zn^{2+} , heme, tropomyosin, heparin, heparan sulphate, plasminogen, and complement (Ronca and Raggi, 2015).

It has been shown that HRG can regulate many biological processes, such as angiogenesis, coagulation, fibrinolysis, and the phagocytosis of apoptotic cells (Poon et al., 2011). Shannon et al. demonstrated that HRG decreased the mortality of a septic mouse model with

S. pyogenes-induced abscesses by killing and trapping of bacteria in the abscess sites (Shannon et al., 2010). Wake et al. demonstrated that HRG prevents septic lethality through the regulation of neutrophils and the inhibition of the strong attachment of neutrophils to vascular endothelial cells (Wake et al., 2016). In recent clinical studies, HRG was further proposed as a new biomarker to predict the outcome of sepsis patient (Kuroda et al., 2018; Nishibori et al., 2018). Based on these findings, our present research demonstrates for the first time the protection effects of HRG on the endothelium, especially the barrier dysfunction of endothelium in the systemic septic condition and the possible mechanism. This will provide new evidence for the supplementary therapy with HRG for the treatment of sepsis.

Methods

Cell culture

EA.hy 926 cells (ATCC Cat# CRL-2922, RRID:CVCL_3901), a hybridoma of human umbilical vein endothelial cells (HUVECs) and the human epithelial cell line A549 were cultured using Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Gibco), 10% L-glutamine (#G7513, Sigma), 5% penicillin/streptomycin (Gibco) in 5% CO₂ at 37 °C. After reaching confluence, the endothelial cells were detached from culture flasks using 0.25% Trypsin-EDTA (Gibco), washed, and resuspended in DMEM. These cells were passaged every 3–4 days, and all experiments were performed with cells kept in culture between three and six passages.

Purification of HRG from human plasma

HRG was purified from human plasma by our lab as described (Mori et al., 2003). Human plasma was supplied by the Japanese Red Cross Society from the healthy volunteer's donation. The study protocol complied with the principles outlined in the Declaration of Helsinki and all subjects signed an informed consent. Briefly, human plasma was incubated with nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) for 2 h at 4 °C with

gentle shaking. The gel was packed into a column and washed successively with 10 mmol/L Tris-buffered saline (TBS) (pH 8.0) containing 10 mmol/L imidazole and then 10 mmol/L Tris-buffer (TB) (pH 8.0) containing 1 mol/L NaCl. Human HRG was eluted by 0.5 mol/L imidazole in 10 mmol/L TBS (pH 8.0). The protein eluate from Ni-NTA was further purified by a Mono Q column (GE Healthcare, Little Chalfont, UK) with NaCl gradient. Purified human HRG was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with a human HRG-specific antibody.

Immunostaining assay

EA.hy 926 cell suspensions (5×10^5 cells/ml) were cultured in 96-well plates (Falcon, Tewksbury, MA) until confluent. The monolayer was then washed with phosphate-buffered saline (PBS) and pretreated with HRG or human serum albumin (HSA) (1 μ mol/L) for 30 min before the stimulation with lipopolysaccharide ([LPS](#)) (*Escherichia coli* 0111:B4, Sigma) or tumor necrosis factor- α (TNF- α) (Sigma-Aldrich) (100 ng/ml). After incubation, the cells were washed with PBS twice, fixed with 4% paraformaldehyde (Wako Japan). Cell cytoskeleton F-actin was stained with Phalloidin-Alexa 568 (Invitrogen, Carlsbad, CA) at room temperature (RT) for 1 h, and the intercellular junction proteins or NF- κ B were stained with primary antibodies to VE-cadherin (Abcam, #ab33168, RRID:AB_870662), β -catenin (Abcam, #ab16051), or NF- κ B p65 (Abcam, #ab16502, RRID:AB_443394) for 1 h at 37 $^{\circ}$ C, followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) secondary antibody for 1 h at RT. Nuclei were stained with DAPI for 5 min. The samples were observed using a confocal microscope (LSM 780, Carl Zeiss).

Western blotting

Cells cultured in six-well plates were collected with RIPA lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1mM DTT, 20mM β -glycerophosphate and protease/phosphatase inhibitors added immediately before

use) and then electrophoresed on polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 10% skim milk for 1 h and incubated overnight at 4 °C with rabbit anti-VE-cadherin Ab, anti-p-VE-cadherin Ab (Abcam, #ab119785), anti-FAK Ab (Abcam, #ab40794, RRID:AB_732300), anti-p-FAK Ab (Abcam, #ab81298), anti-p38 Ab (Cell Signaling Technology, #9212S, RRID:AB_330713), anti-ERK1/2 Ab (Cell Signaling Technology, #12940), anti-p-p38 (T180/Y183, Cell Signaling Technology, #4631SS, RRID:AB_331765) or anti-p-ERK1/2 Ab (T202/Y204, Cell Signaling Technology, Cat# 4370, RRID:AB_2315112), and anti-MLC and anti-p-MLC Ab (Abcam, #ab157747) followed by goat anti-rabbit IgG-HRP (MBL, Nagoya, Japan) for 2 h at RT. The signals were visualized by the luminal-based enhanced chemiluminescence (ECL) HRP substrate method (Thermo Fisher Scientific, MA). An Image Quant LAS4000 system was used for detection, and images were analyzed with ImageJ software ver. 1.51.

Cell detachment assay

Firstly, 96-well plates were coated with 5 µg/well extracellular matrix protein-laminin (Sigma-Aldrich) at 37 °C for 2 h, then washed twice with PBS. EA.hy 926 endothelial cell suspensions were added into plain or laminin-coated plates together with the mixture of HRG or HSA and LPS or TNF- α (100 ng/ml). After incubation for 2 h, the detached cells were washed out with PBS, and the adherent cells were fixed with 4% paraformaldehyde for 30 min and stained for 15 min with 1% crystal violet (Wako, Osaka, Japan). The cells were washed twice with PBS and air-dried and then observed with an All-in-one Fluorescence Microscope (BZ-X700, Keyence, Japan). After the microscopic observation, Crystal violet was extracted from the cells with 100% methyl alcohol, and optical density was measured at 595 nm wavelength with a multi-detection reader Flex Station 3 (Molecular Devices, CA).

Cytometric bead array (CBA)

We examined the secreted cytokines in the supernatant of cultured endothelial cells by performing a cytometric bead array using a Human Soluble Protein Master Buffer Kit and cytokine Flex Set (BD Biosciences, CA) following the manufacturer's instructions. Generally, multiple capture beads for IL-6, [IL-8](#), [IL-1 \$\alpha\$](#) and [IFN- \$\gamma\$](#) were mixed together. The mixed capture beads were co-incubated with 50 μ l of supernatant and detection reagent for 2 h. The beads were then washed carefully and resuspended. Samples were analyzed using a FACSCanto II system (BD Biosciences). The data were analyzed with FCAP Array software.

RNA-Isolation and Real-Time PCR

Cells were harvested and mRNA was extracted using an RNeasy mini kit (Qiagen). Complementary DNA was synthesized with a Takara RNA PCR kit ver. 3.0 (Takara Bio, Nagahama, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed with a Light Cycler (Roche, Switzerland) according to the manufacturer's instructions. The primers shown in supplementary table were used to amplify specific cDNA fragments. β -actin expression was used to normalize cDNA levels. The PCR products were analyzed by a melting curve to ascertain the specificity of amplification and the relative fold gene expression of samples were calculated with the delta-delta Ct method.

In vitro permeability assay

EA.hy 926 endothelial cells were seeded at a density of 5×10^5 cells/ml onto 24-well plates with 6.5-mm-dia. transwell inserts and a 0.4- μ m pore size polyester membrane (Corning, MA) and cultured until confluent. Cells were pre-incubated for 30 min with HRG/HSA before stimulation with LPS/TNF- α . After 12 h, the culture medium was replaced with medium containing 0.5 mg/ml FITC-dextran (70 kDa, 150kDa, 250kDa, Sigma-Aldrich) in the upper chamber. The fluorescence in the lower chamber, which represents the permeability of the monolayer of endothelial cells, was detected with the Flex Station 3 (Ex = 492 nm, Em = 518 nm) at different time points.

Animals

Adult male C57BL/6N mice (22±3 g, 8 week, RRID: MGI_5658420) were purchased from SLC (Hamamatsu, Japan) and then housed in the Okayama University institutional animal units (12 h light cycle). The mice were maintained on a standard rodent diet with free access to water. Up to four mice were kept per plastic cage with aspen wood bedding material. All procedures involving animals were performed in accordance with guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the ethics review board of the Okayama University. Every effort was taken to minimize the number of animals used and their suffering (in line with the 3Rs).

Randomization and blinding

Animals were randomized for treatment. Data collection and evaluation of all experiments were performed blindly of the group identity. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018).

Sepsis model

Two types of septic animal models were used in the present study: an LPS-induced endotoxemia model and a cecal ligation puncture (CLP) model. The LPS-induced septic model was established with an intravenous injection of LPS (10 mg/kg) and was used for experiments 12 h thereafter (Xu et al., 2015; London et al., 2010). The CLP septic animal models were established as described in our previous study (Wake et al., 2016). Briefly, the mice were anesthetized with (3% isoflurane in 48.5% N₂O and 48.5% O₂) and a ligature was set below the ileocecal valve. The cecum was gently exteriorized from the peritoneal cavity and punctured twice with an 18-ga. needle and then was returned back to the abdomen. The mice were sacrificed 24 h later with pentobarbital sodium (50 mg/kg, i.p.).

Vascular permeability in septic mice

We assessed the vascular permeability of different organs in endotoxemic or septic mice by determining the Evans Blue leakage from capillary. First, HRG or HSA in vehicle (PBS) was administered through the tail vein immediately after the operation. Each mouse was given 20 mg/kg HRG or HSA in a volume of 200 μ l (i.v.). After 12 h or 24 h, mice were given an intravenous injection of Evans blue albumin (EBA, 20 mg/kg, Wako). EBA was allowed to circulate for 1 h, and the mice were then anesthetized and perfused with saline. The lungs, liver and kidney were excised, weighed, homogenized, and kept in 2 ml of formalin for 48 h at 60 $^{\circ}$ C. The extracted dye was then measured with a spectrometer at 610 nm. The absorbance value was converted to micrograms of Evans blue dye per gram wet weight of lung, liver or kidney, respectively (London et al., 2010).

Rho GTPase pull-down assay

Rho A regulates molecular events by cycling between an inactive GDP-bound form and an active GTP-bound form. In this study, RhoA GTPase activity was detected by a Rho GTPase pull-down assay for the GTP-bound active RhoA according to the manufacturer's instructions (Cell Biolabs, STA-403-A-T, CA). EA.hy 926 cell suspensions (5×10^5 cells/ml) were cultured in 6-well plates until confluence. The cells were then washed with PBS and pretreated with HRG or HSA (1 μ mol/L) for 30 min before the stimulation with LPS for 1h. At the end of the incubation, the culture medium was removed and the cells were washed with ice-cold PBS. Then, the cells were detached from the plate by adding ice-cold 1X Lysis buffer (Part No. 240102-T) and scraping with a cell scraper. Cell lysates were then incubated with agarose beads containing Rhotekin-binding domain to pull down the activated RhoA. The beads were collected, and proteins were analyzed by immunoblotting.

Statistical analysis

Data were analysed with GraphPad Prism statistic software (version 6.01, San Diego, CA). All values are presented as the means $\pm$ SEM and analysed by ANOVA followed

by Bonferroni's test or *post hoc* Fisher test when the F statistic was significant. $P < 0.05$ was considered statistically significant. At least three independent experiments were performed with all the assays.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

HRG inhibited stress fiber formation and cytoskeleton reorganization

Given the well-known important functions of actin and actin-binding proteins for endothelial barrier homeostasis (García *et al.*, 2015), we investigated the effects of HRG on the stress fiber formation and cytoskeleton remodeling induced by LPS/TNF- α in endothelial cells. Under the resting condition, F-actin predominated as a thin band along the cell boundaries, and no F-actin bundles were present inside the cells (Fig. 1A, left panel). We observed that 10 ng/ml of LPS/TNF- α stimulation for 2 h induced the reorganization of the actin cytoskeleton, as evidenced by an increase in the thickness of the peripheral F-actin band and the appearance of F-actin bundles (stress fibers, indicated by arrows in Fig. 1A, middle panel) inside the cell. Our quantitative analysis of these actin structures by determining the pixel intensities of these fibers inside the cells (Fig. 1B, upper panel) revealed a significant increase in such F-actin fibers compared to the control group (Fig. 1C, left panel). Additionally, the fluorescence intensity of the horizontal diameter of the cell shown in Figure 1B (lower panel, red line) was measured as the central stress fiber density as described by Peacock (Peacock *et al.*, 2007). These measurements invariably revealed a significant increase in actin fibers crossing the cell centers

of the cells stimulated with LPS/TNF- α (Fig. 1C, right panel).

After the cells were stimulated with LPS/TNF- α (100 ng/ml) for 12 h, we found that F-actin had broken down and redistributed at the cell periphery accompanied by cell contraction toward the cell center (Fig. 1D, middle panel). The results were quantified with the cell area shown in Figure 1E. HRG (1 μ mol/L) effectively inhibited this stress fiber formation (Fig. 1A, right panel) and cell morphological changes (Fig. 1D, right panel) and maintained the monolayer integrity. The results also demonstrated the concentration-dependent effects of HRG on the morphology changes induced by LPS (Suppl. Fig. S1). HRG (1 μ mol/L) significantly inhibited the phosphorylation of myosin light chain induced by LPS/TNF- α (Figure. 1F). The quantification of western blotting results is illustrated in Figure 1G. These results suggested that HRG inhibited the stress fiber formation and cytoskeleton reorganization through the regulation of myosin light chain phosphorylation. However, HSA, a major plasma protein, did not show any inhibitory effects at the same concentration (1 μ mol/L). The inhibitory effects of HRG on the LPS/TNF- α induced cytoskeleton reorganization and morphology changes are also true for the primary culture of human lung microvascular endothelial cells (HMVECs) (Suppl. Fig. S6).

HRG prevented the loss of intercellular adherent junctions

VE-cadherin is a major transmembrane adherent junction protein in vascular endothelial cells, and it represents a crucial determinant of endothelial barrier integrity. The immunostaining of VE-cadherin and β -catenin showed a linear staining pattern at the cell borders under control conditions in confluent endothelial monolayers, (Fig. 2A,B, left panel). Incubation with LPS/TNF- α for 6 h led to intercellular gap formation and a pronounced loss of VE-cadherin and β -catenin staining at the cell borders (middle panel). Pretreatment with HRG significantly prevented the loss of both VE-cadherin and β -catenin (Fig. 2A,B right panel). The inhibitory effects of HRG on the loss of VE-cadherin also showed dose-dependency (Suppl. Fig. S2). These protective effects are also true for the HMVECs (Suppl. Fig. S7). Scanning electron microscopy observations also confirmed the formation of intercellular gaps, thickness of the cell

membrane and microparticle-like structure on the cell surface induced by LPS; this was also inhibited by HRG (Fig. 2C). In parallel, the western blotting results of VE-cadherin also showed that HRG prevented the decrease in the expression of VE-cadherin induced by LPS or TNF- α (Fig. 2D,E). The phosphorylation and dephosphorylation of intercellular tyrosine residues in the VE-cadherin complex are responsible for the modulation of VE-cadherin function (Weidert et al., 2014). Wessel et al. showed that Tyr 685 and Tyr 731 of VE-cadherin distinctly and selectively regulate the induction of vascular permeability or leukocyte extravasation (Wessel et al., 2014). In this study, we investigated the effects of HRG on the Y-685 phosphorylation in VE-cadherin. The results showed that HRG effectively inhibited the LPS/TNF- α -stimulated phosphorylation of VE-cadherin at Tyr 685 site (Fig. 2D, E).

Moreover, HRG prevented the LPS/TNF- α -induced loss of other junction molecules like occludin and ZO-1 which were involved in the endothelium permeability control (Suppl. Fig. S3).

LPS/TNF- α -induced degradation of FAK and cell detachment were reduced by HRG

Focal adhesion kinase (FAK) is associated with actin stress fibers, which serve as holding points for cytoskeletal tension. FAK is responsible for the regulation of cell contractility and cell-matrix adhesion. LPS was reported to induce the stress fiber formation, associated with phosphorylation and degradation of FAK. The results in Fig. 3A showed that LPS and TNF- α induced slight FAK degradation and strongly stimulated phosphorylation of FAK, which were inhibited by the treatment with HRG. These results proved that HRG not only prevented the loss of FAK but also effectively regulated the FAK phosphorylation stimulated by LPS/TNF- α . To further confirm the regulation effects of HRG on cell-matrix adhesion, the cell detachment assay was performed. Results showed that LPS/TNF- α stimulation led to a large number of cells detaching from the plain plates (Fig. 3B-a,c) and laminin-coated plates (Fig. 3B-b,d) whereas HRG effectively prevented the cell detachment under both conditions. The results also statistically analyzed by the measurement of optical density of crystal violet remaining on the

plates at 595 nm (Fig. 3C).

HRG suppresses the cytokine secretion and mRNA expressions of inflammation-related molecules in endothelial cell

According to the results, 12 h incubation of endothelial cells with LPS/TNF- α resulted in a large increase in the secretion of IL-6, IL-8, IL-1 α , and IFN- γ in the culture media compared to the non-stimulated control group. HRG (1 μ mol/L) almost completely inhibited all of the cytokine release induced by LPS, and the same concentration of HRG had less effect on the IL-6 release induced by TNF- α (Fig. 4A). Consistent with above results, HRG also reduced the expression of IL-6, IL-8, IL-1 α , IFN- γ and [IL-10](#) at the mRNA levels (Fig. 4B). From these results, we speculate that the reduced cytokine secretions from endothelial cells by HRG were due to the regulation of both secretory pathway and mRNA expression.

Moreover, our previous study (Wake et al., 2016) already proved that the systemic injection of HRG significantly reduced the cytokine levels in the septic mice compare with control HSA-injected group. In the present study, the effects of HRG on the serum cytokine levels in LPS-injected mice were also determined with CBA. HRG significantly reduced the IL-6, IL-10, and TNF- α levels in serum of LPS-injected mice compare with control HSA-injected group (Suppl. Fig. S4).

As presented in Figure 4B, LPS enhanced the expression of all three receptors ([TLR2](#), [TLR4](#), and [RAGE](#)) on endothelial cells. Interestingly, HRG partially but significantly inhibited the stimulatory effects of LPS on the expression of all three receptors. The stimulatory effect of LPS on the expression of TLR2 was higher than those of the other two receptors, and the inhibitory effect of HRG on the expression of this receptor was also more pronounced. MMP-2 has been reported to be secreted from different cells (Alexander et al., 2002), leading to the degradation of tight junctions and adherens junctions which results in an increase in the vascular permeability. The results also showed that HRG can effectively suppress the LPS-induced up-regulation of MMP-2 mRNA.

LPS/TNF- α -induced NF- κ B activation were prohibited by HRG in immunostaining

NF- κ B/p65 is a member of the NF- κ B protein family. It is transferred to the nucleus and bound to a specific sequence in the genome promoter regions, resulting in the regulation of proinflammatory responses and endothelial permeability when the cells receive inflammatory stimuli (Sprague and Khalil, 2009). We observed that NF- κ B/p65 was located in the cytoplasm in the non-stimulated control group, whereas in the LPS/TNF- α treated cells, NF- κ B/p65 was translocated into the nuclei. LPS-induced changes in translocation occurred 12 h after stimulation (Fig. 5A), and the translocation by TNF- α was evident 6 h after stimulation (Fig. 5B). However, pretreatment with HRG clearly suppressed the LPS/TNF- α -induced NF- κ B/p65 translocation to nuclei in endothelial cells (Fig. 5A,B). The results of our quantitative analysis of the relative intensity of fluorescence in the nuclei/cytosol are provided in Figure 5C. The preventive effects of HRG on the LPS/TNF- α -induced NF- κ B activation were also observed in HMVECs (Suppl. Fig. S8).

Protective role of HRG on the permeability of vascular endothelium in vitro and in vivo

In the FITC-dextran transwell assay as depicted in Figure 6A, LPS/TNF- α -induced significant leakage of 70kDa FITC-dextran from the endothelial monolayer compared to the control group, and pretreatment with HRG produced a significant inhibition of dextran leakage during the 12h stimulation compared to the HSA-treatment group. This effect was also true for LPS-induced 150kDa or 250kDa FITC-dextran leakage (Fig. 6B, C). Moreover, HRG also showed the inhibitory effects on the LPS/TNF- α -induced hyperpermeability in HMVEC monolayer in vitro (Suppl. Fig. S5).

To examine whether HRG reduces hyperpermeability under cytokine storm conditions in vivo, we used a bacterial endotoxin model of inflammation. LPS administration triggered a large increase in capillary permeability at 12 h after the injection. Using EBA as a tracer, we observed that HRG significantly reduced the EBA leakage from capillaries in the lung and liver of LPS-injected mice (Fig. 6D). To test the physiological relevance of our findings and to

determine whether the effect of HRG is limited to the administration of LPS, we used a model of polymicrobial sepsis known as cecal ligation and puncture (CLP). HRG also significantly reduced vascular permeability in the lung, liver and kidney after 24 h in the sepsis-induced barrier dysfunction model (Fig. 6E).

HRG prevented the activation of MAPK and Rho signal pathway induced by LPS

Rho-associated kinase (ROCK) and mitogen-activated protein kinase (MAPK) cascades, which include p38 and ERK1/2, play critical roles in the regulation of VE-cadherin redistribution and cytoskeletal polymerization in endothelial permeability control (Yuan et al., 2002). The results show that LPS stimulation induced p38 and ERK1/2 phosphorylation in a time-dependent manner, as the p38 and ERK1/2 activations were biphasic with an initial increase (peaking at 1 h) followed by a slight drop in the phosphorylation levels (Fig. 7A,B). HRG effectively inhibited the phosphorylation of p38 and ERK1/2 induced by LPS (Fig. 7C). The inhibitory effect of HRG on the LPS-induced activation of Rho A (GTP-bound) was shown in Figure. 7D. The quantification of western blotting results is illustrated in Figure 7E.

Discussion

Endothelial barrier dysfunction and microvascular leakage critically contribute to the pathogenesis of organ failure and sepsis-related complications (Goldenberg et al., 2011). The endothelium is thus generally regarded as a target for the treatment of sepsis, especially concerning endothelial barrier repair strategies. Currently there is no appropriate therapy for recovery the endothelial barrier dysfunction in sepsis. The present study extends our previous investigations and shows that plasma protein HRG has potent protection effects on the LPS-or septic-induced barrier dysfunction of endothelium.

The vascular barrier function is maintained by the balance between endothelial cells' contractile forces and adhesive cell-cell and cell-matrix tethering forces; the former generate centripetal tension, and the latter regulate cell shape. Stress fibers composed of bundles of

polymerized actin are the primary elements of the contractile machinery of endothelial cells, which assembled in a characteristic manner in response to permeability-increasing mediators. In our experiments, the stimulation with a lower concentration (10 ng/ml) of LPS/TNF- α for 2 h induced the stress fiber formation in endothelial cells (Fig. 1A), whereas a higher concentration of LPS/TNF- α (100 ng/ml) for 12 h resulted in marked changes in cell morphology (Fig. 1D). These results suggested a time- and dose-dependent increase in cytoskeleton rearrangement and contractile forces. HRG effectively inhibited the stress fiber formation and cell rounding, thus maintaining the monolayer integrity in a dose-dependent manner (Suppl. Fig. S1). Phosphorylation of MLC (p-MLC) enables the formation of stress fiber and leads to increased contractility in endothelial cells (Nwariaku et al., 2002; García Ponce et al., 2016). The results in Fig. 1F and G showed that HRG effectively reduced LPS/TNF- α -induced phosphorylation of myosin light chain indicating that HRG's protective effects on endothelial barrier function are related to the prevention of contractile stress fiber formation through the inhibition of phosphorylation of MLC.

VE-cadherin is the major transmembrane intercellular adherens junction that links to the actin cytoskeleton through the α -catenin and β -catenin to provide both mechanical stability and the transduction of extracellular signals into the cell, and it thus represents a crucial determinant of endothelial barrier integrity (Dejana and Orsenigo, 2013). Soluble VE-cadherin appears to play a role in sepsis and has the potential to be a clinical marker for the early detection of the loss of microvascular barrier functions in sepsis (Flemming et al., 2015). Focal adhesions provide additional adhesive forces in barrier regulation by forming a critical bridge for bidirectional signal transduction between the actin cytoskeleton and the cell-matrix interface. FAK is a major contributor to the regulation of focal adhesion formation and is a key determinant of vessel wall permeability through the regulation of cell-matrix adhesion. Our results demonstrated that HRG inhibited the loss of VE-cadherin and β -catenin at the cell border (Fig. 2A, B) and also prevented the FAK degradation and phosphorylation induced by LPS/TNF- α (Fig. 3A). In addition, HRG enhanced the cell-matrix adhesion by inhibiting the cell detachment from

laminin-coated plates (Fig. 3B,C). These results revealed that HRG protected the permeability of vascular endothelial cells through the regulation of intercellular junctions as well as cell matrix adhesion. Two phosphorylation sites of VE-cadherin (Tyr 731 and Tyr 685) have been reported to be involved in controlling endothelial permeability and regulation of cell-cell adhesions (Weidert et al., 2014). In fact, it was found that HRG inhibited the phosphorylation of VE-cadherin at Tyr 685. This mechanism may contribute to the maintenance of the intercellular junctions.

During the septic process, many pathogenic factors can stimulate the production of inflammatory mediators that lead to microvascular dysfunction. LPS acting on the endothelial cells can facilitate the release of secondary mediators such as TNF- α , IL-1 α , and IL-8 and the activation of toll-like receptors. Thus, the inhibition of the production of pro-inflammatory cytokines may be a key factor in the prevention and therapy of sepsis. In present study, we examined the secretion of IL-6, IL-8, IL-1 α , and IFN- γ and the expression of mRNAs for TLR2, TLR4, RAGE, MMP-2, IL-6, IL-8, IL-1 α , IFN- γ and IL-10 in LPS-induced endothelial cells. The results presented in Figures 4 demonstrated that pretreatment with HRG can strongly inhibit the enhanced expression/secretion of cytokines as well as pattern recognition receptors. IL-8 is a well known neutrophil chemotactic factor. The inhibitory effects of HRG on the IL-8 secretion and expression also supported the previous finding that HRG can strongly suppress the adhesion of neutrophils to lung vasculatures in septic mice (Wake et al., 2016). Moreover, the suppressive effects of HRG on hypercytokinemia in septic mice were also observed in the LPS-injected mice (Suppl. Fig. S4). These findings support the hypothesis that HRG may exert anti-septic effects through the regulation of cytokine production and barrier dysfunction in vascular endothelial cells, in addition to a strong regulator role for neutrophils (Wake et al., 2016).

LPS activates the release of these cytokines by a transcriptional mechanism involving the activation of NF- κ B (Cohen J, 2002). NF- κ B is an important transcription factor involved mainly in inflammatory responses. Our immunofluorescence staining analysis revealed that HRG suppressed the translocation of NF- κ B (p65) from the cytoplasm into the nucleus induced

by LPS/TNF- α (Fig. 5), which further explains the effective role of HRG on inhibition of cytokine secretion induced by LPS.

Our results showed that HRG prevented the LPS/TNF- α -induced FITC-dextran leakage with different size from the endothelial monolayer, even after 12-h stimulation (Fig. 6 A-C). The fluorescence intensity of 70 kDa FITC-dextran in lower chamber of vascular endothelial monolayer stimulated by LPS is higher than that of 150 kDa and 250 kDa. In fact, with the prolongation of the stimulation time, the endothelial monolayer seemed to spontaneously develop FITC-dextran leakage. This may be ascribed to the usage of fetal bovine serum-free medium for the cell culture. Even under such conditions, HRG had a significant inhibitory effect on the permeability compared to the HSA-treated group, but HRG had no effect on the basal permeability.

HRG also prevented the hyperpermeability of different organs, lung, liver and kidney in the LPS-injected mice and septic mice (Fig. 6 D,E). Wake et al. demonstrated that HRG treatment inhibited the leukocyte infiltration and edema in lung and glomerulus in CLP mice at 24 h after the onset (Wake et al., 2016). Thus, the present results are consistent with the previous findings concerning the protection of capillary permeability by HRG.

In our experiment, EA.hy 926 immortalized endothelial cell line was used for the study of the effects of HRG on the barrier dysfunction induced by LPS/TNF- α . To further confirm the role of HRG on the endothelial permeability, we also performed several experiments with culture of primary human lung microvascular endothelial cells (HMVECs) which was often used for the permeability study. The results showed that HRG have potent inhibitory effects on the LPS/TNF- α -induced hyperpermeability, cytoskeleton reorganization, loss of VE-cadherin and NF- κ B activation (Suppl. Fig. S5-S8) in HMVECs, which provided critical evidence that HRG produces protective effects on the barrier function under inflammatory conditions both in EA.hy 926 endothelial cells and HMVECs.

To elucidate the potential mechanisms leading to the inhibition of NF- κ B activation by HRG, we explored LPS-induced signaling cascades including two distinct types of MAPKs (p38

MAPK and ERK1/2). The two MAPKs are known to act as upstream intermediates providing a link between extracellular stimuli and cytokine production in various cell lines. Once activated, both p38 MAPK and ERK1/2 may activate transcription factors such as NF- κ B (Johnson and Lapadat, 2002; Hippenstiel et al., 2000). In addition, both p38 MAPK and ERK1/2 are involved in the regulation of cytoskeleton rearrangement and the permeability control of endothelial cells. We observed a time-dependent activation of p38 and ERK1/2 by LPS with the maximal activation at 1 h. This transient activation of MAPKs was inhibited by the treatment with HRG, whereas HSA had no such effects (Fig. 7A-C). A key contractile event in endothelial cells is the phosphorylation of regulatory MLC catalyzed by Ca^{2+} /calmodulin-dependent MLC kinase and/or through the activation of the Rho/Rho kinase pathway. It has been known for several years that RhoA and ROCK activation are present downstream of many permeability-increasing mediators and contribute to increased permeability. Our results also demonstrated that HRG inhibited the activation of RhoA which explains the manner in which HRG may inhibit the morphology changes and hyperpermeability of endothelial cells stimulated by LPS/TNF- α (Fig. 7D).

Clinical study also performed to collect blood samples from patients within 24 hours of ICU admission and HRG levels were measured with enzyme-linked immunosorbent assay. The results showed that plasma HRG levels of the septic patients (8.71 $\mu\text{g/mL}$) were significantly lower than in healthy volunteer (63.0 $\mu\text{g/mL}$), and also lower than in noninfective systemic inflammatory response syndrome patients (33.3 $\mu\text{g/mL}$) (Kuroda et al., 2018; Nishibori et al., 2018). Histidine-rich glycoprotein showed a high sensitivity and specificity for diagnosing sepsis. Moreover, plasma HRG levels were a good prognostic indicator and the determination of plasma HRG alone could still predict mortality (Kuroda et al., 2018; Nishibori et al., 2018). Our present research proved that HRG has potent protection effect on vascular endothelium barrier under septic condition, which provides the evidence for the supplementary treatment of sepsis with HRG, however, the specific mechanism of HRG on the protection of endothelium need to be further investigated.

Conclusion

The present study provided strong evidences that HRG efficiently protected vascular endothelial cells under inflammatory conditions. The effects may contribute to anti-septic effects of HRG administered systemically. Thus, the present study not only clarifies the effects of HRG on vascular endothelial cells, but also facilitates our understanding of sepsis pathophysiology. As a whole, the supplementary therapy with HRG should be persuaded for the treatment of sepsis

Author contributions

S.G conceived the study, designed the experiments, analysed data and wrote the manuscript. M. N and H.W for editing the manuscript; S.M and H.W for the purification of HRG from human plasma; Y.G, D.W and K.L for performing the experiments; K.T and H.T for critically reviewing the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for [Design & Analysis](#), [Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Legends

Fig. 1. Effects of HRG on LPS/TNF- α -induced stress fiber formation and cytoskeleton reorganization. EA.hy 926 endothelial cells were incubated with 1 μ mol/L of HSA, HRG, or PBS for 30 min before stimulation with 10 ng/ml LPS/TNF- α for 2 h (**A**) or 100 ng/ml for 12 h (**D**). The actin distribution and morphology of endothelial cells were analyzed by fluorescence using Alexa 568-phalloidin. Actin staining is in gray (A) or red (D), nucleus staining is blue. Images are representative of three independent experiments. Bar = 20 μ m. **B:** *Upper:* The method of actin fiber quantification as depicted as the yellow arrow shown in (A). *Lower:* The assessment of central actin fiber density, the results of which are shown in (C). Pixel intensities (C) and cell areas (E) were quantified using ImageJ software. (F) Western blot results of HRG on the LPS/TNF- α -induced phosphorylation of MLC. (G) The quantification of western blotting results in Fig. 1F. The results in graphs are shown in the means $\pm$ SEM (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Fig. 2. HRG prevents the loss of intercellular junction. Immunostaining results of VE-cadherin/ β -catenin in endothelial cells stimulated with 100 ng/ml LPS or TNF- α for 6 h after treatment with HRG or HSA. **A,B:** Cells were incubated with anti-VE-cadherin mAb or anti- β -catenin mAb for 2 h and then stained with Alexa Fluor 488 (green) goat-anti-rabbit IgG. The cells were also stained with DAPI (blue) to visualize the nuclei. The pictures are representative from three independent experiments (n=5 per group). Scale bar = 20 μ m. **C:** SEM pictures of vascular endothelial cells. Scale bar = 50 μ m (*upper panel*) or 20 μ m (*lower panel*). **D,E:** Quantification results of the p-VE-cadherin and VE-cadherin by western blotting. One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Fig. 3. Effects of HRG on the FAK degradation and cell detachment. **A:** Western blot results

and quantification of the phosphorylated FAK (p-FAK) and degraded FAK induced by LPS/TNF- α with Image J. **B:** The cell suspensions were plated into 96-well plain plates (**a,c**) or laminin-coated plate (**b,d**). The cell detachment was evaluated by the staining of remaining cells with 1% crystal violet. **C:** Quantitative analysis of crystal violet extracted from the remaining cells. (a–d) correspond to a–d in panel B. Data are means $\pm$ SEM in the graph (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA; Scale bar = 10 μ m.

Fig. 4. HRG inhibited the cytokine secretion and mRNA expressions of inflammation-related molecules in endothelial cells. The cell culture media were collected for the determination of cytokines. **A:** The levels of IL-6, IL-8, IL-1 α , and IFN- γ in the culture media from each group are shown as the means $\pm$ SEM (n=5 per group). **B:** The expressions of TLR2, TLR4, RAGE, MMP-2, IL-6, IL-8, IL-1 α , IFN- γ and IL-10 at the mRNA level on endothelial cells were measured by quantitative RT-PCR. The results were normalized to the expression of β -actin and are expressed as the means $\pm$ SEM (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Fig. 5. HRG suppresses the LPS/TNF- α -induced NF- κ B activation in endothelial cells. Immunostaining results of NF- κ B in endothelial cells stimulated with 100 ng/ml LPS for 12 h or 100 ng/ml TNF- α for 6 h after treatment with HRG or HSA. **A, B:** Cells were stained with anti-NF- κ B/p65 mAb for 2 h and then stained with Alexa Fluor 488 (green) goat-anti-rabbit IgG. Cells were also stained with DAPI (blue) to visualize the nucleus. The results shown are representative of ≥ 5 experiments. Scale bar = 10 μ m. **C:** The nuclei/cytosol fluorescence intensity ratio represents the translocation of NF- κ B from the cytoplasm to nucleus. Data are means $\pm$ SEM (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Fig. 6. Protection effects of HRG on the endothelial permeability in vitro and in vivo. A-C:

Effects of HRG on LPS/TNF- α -induced hyperpermeability of the endothelial monolayer. The graph shows the fluorescence intensity of FITC-dextran leakage with different sizes from transwell filters to the lower chamber. Data are means $\pm$ SEM in the figure (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA. **D:** Mice were given an intravenous injection of LPS (10 mg/kg) and then treated with HRG or HSA (20 mg/kg) for 12 h. **E:** Mice were subjected to CLP or a sham operation and then an immediate intravenous injection of HRG or HSA. At 12h or 24 h later, the mice received an intravenous injection of EBA, and EBA extraction was measured in the lung, liver and kidney to assess vascular permeability (n=6 per group). The extracted dye was then measured with a spectrometer at 610 nm. The absorbance value was converted to micrograms of Evans blue dye per gram wet weight of lung, liver or kidney, respectively. Statistical analysis was performed by One-way ANOVA with Bonferroni's post-hoc test, **p<0.05 vs. sham, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Fig. 7. Effects of HRG on the inhibition of MAPK and Rho activation. The cells were collected after stimulation with LPS at different time point, and a western blot assay was then performed. **A, B:** Time-dependent phosphorylation of p38 and ERK1/2 induced by LPS. **C:** Effects of HRG on the LPS-induced phosphorylation of p38 and ERK1/2 at 1 h. **D:** The effect of HRG on the activation of RhoA was measured at 1 h. **E:** Quantification of the above western blotting results. Data are means $\pm$ SEM in the graph (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Figure. 1

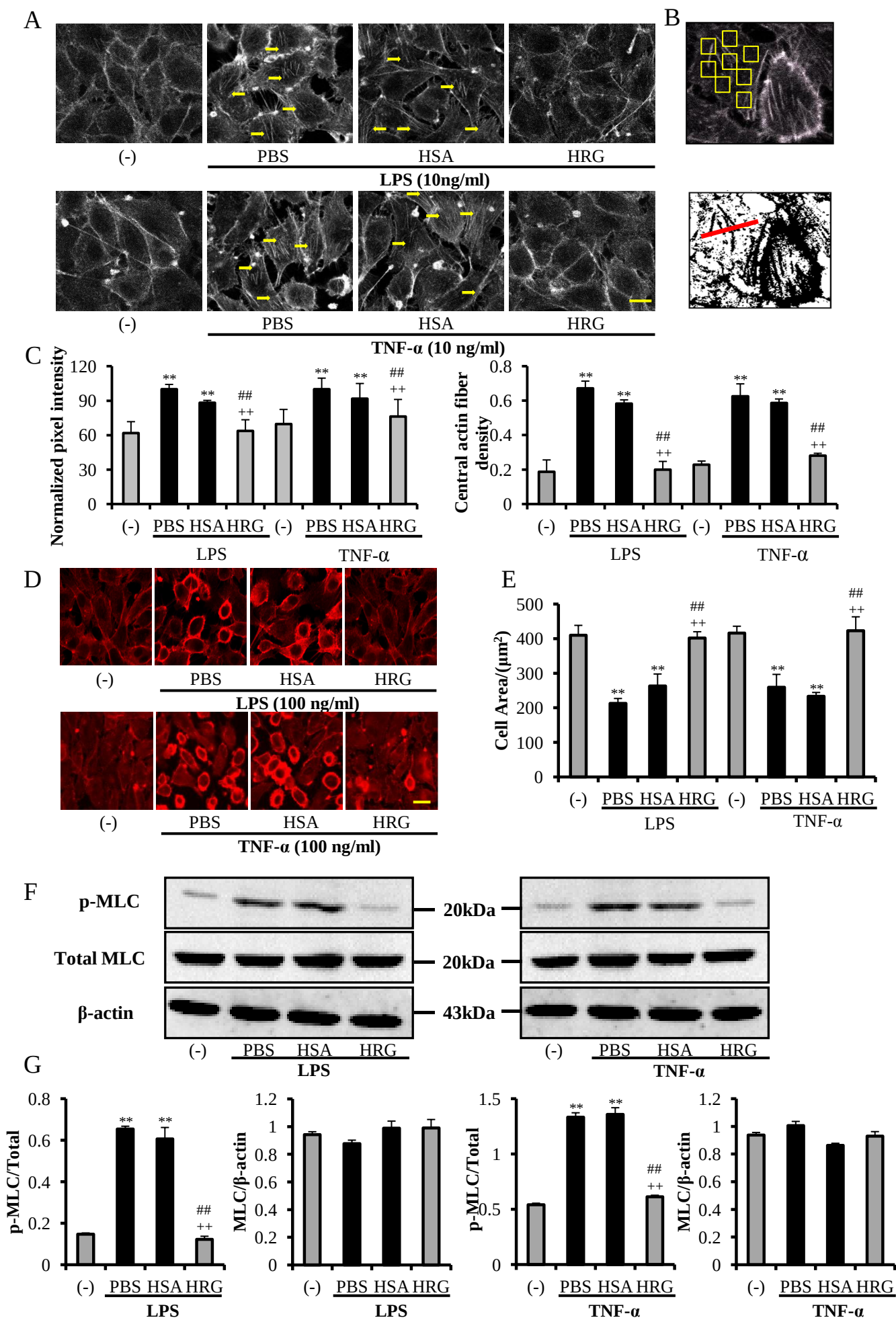


Figure. 2

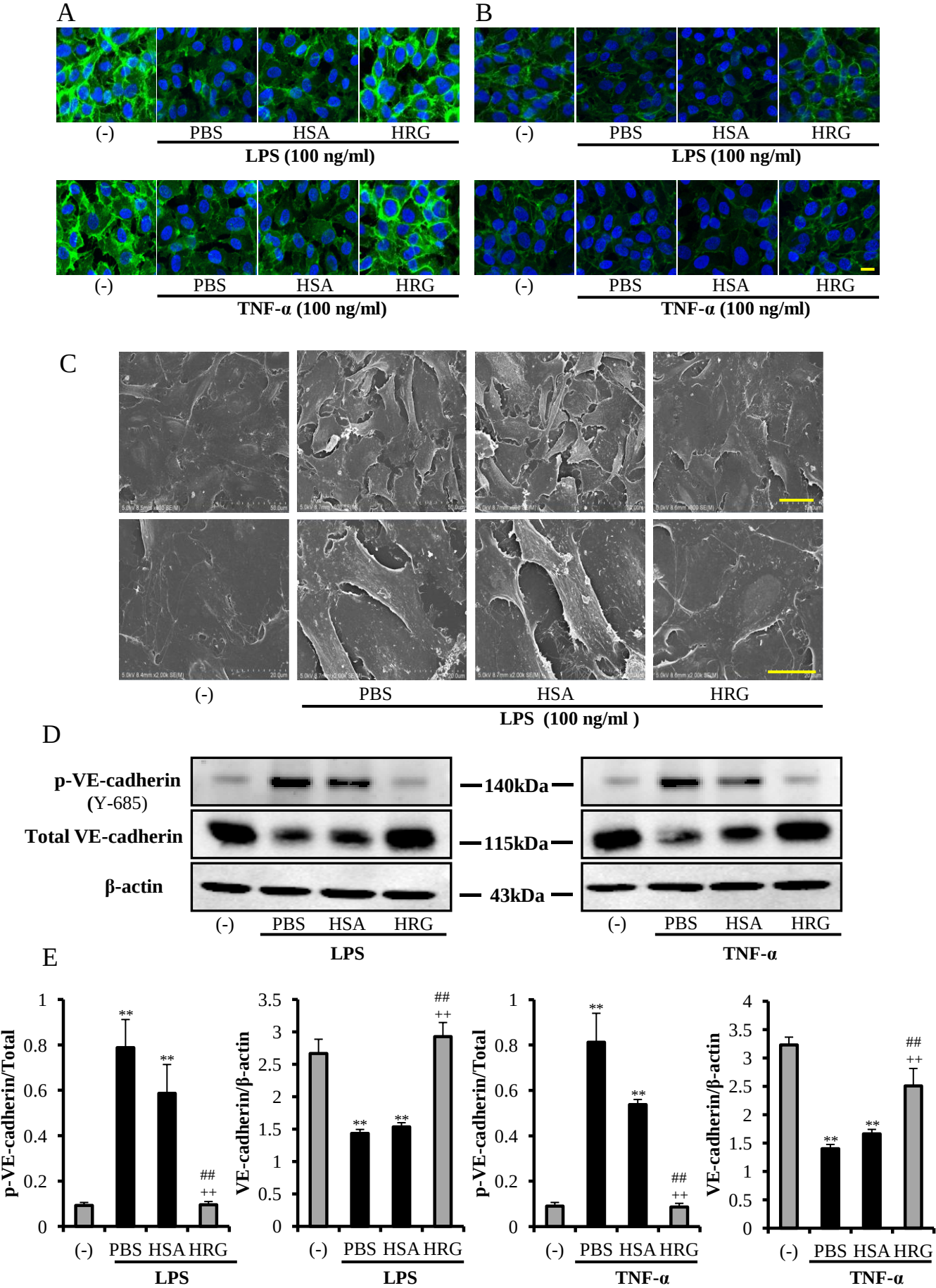


Figure. 3

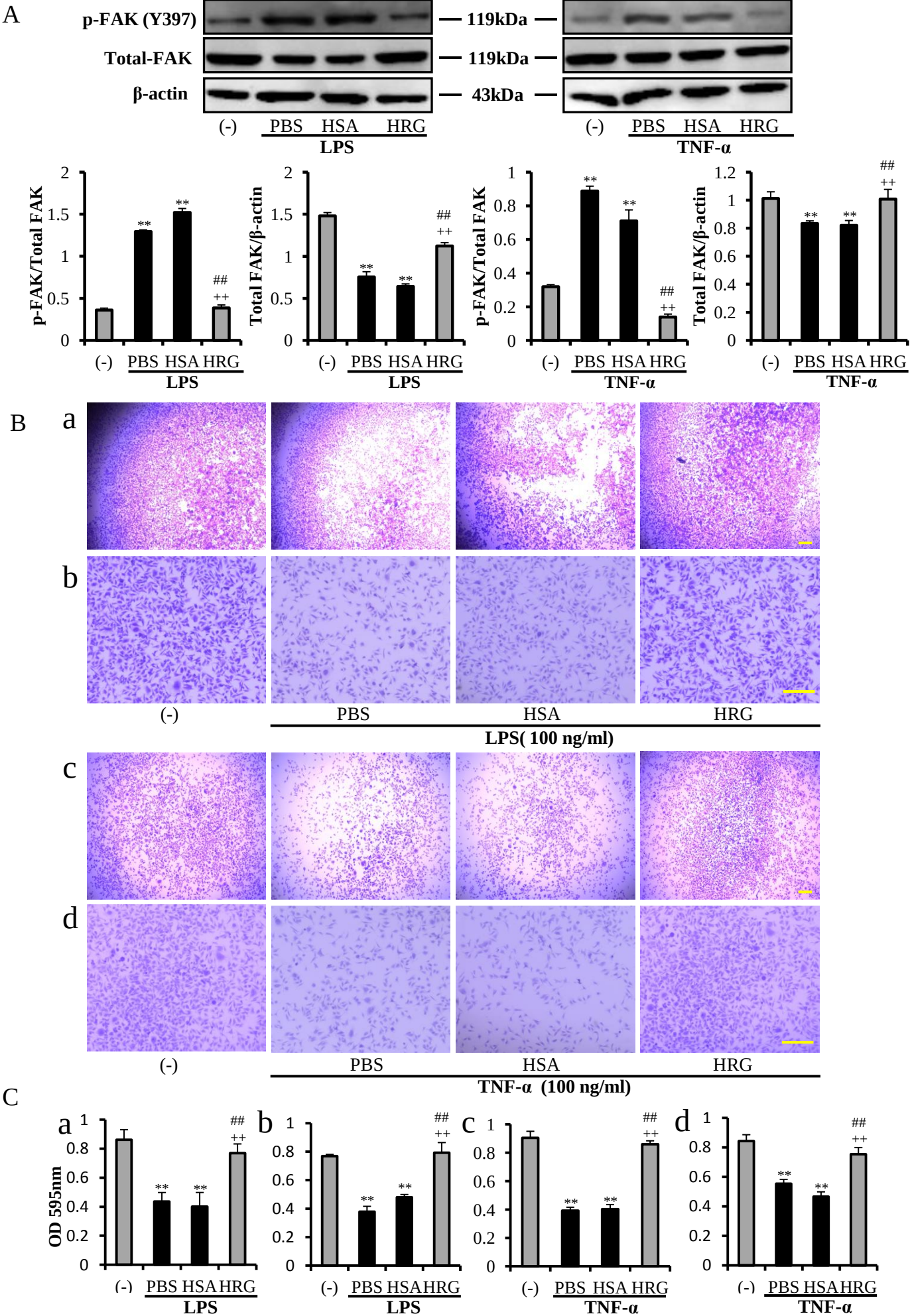


Figure. 4

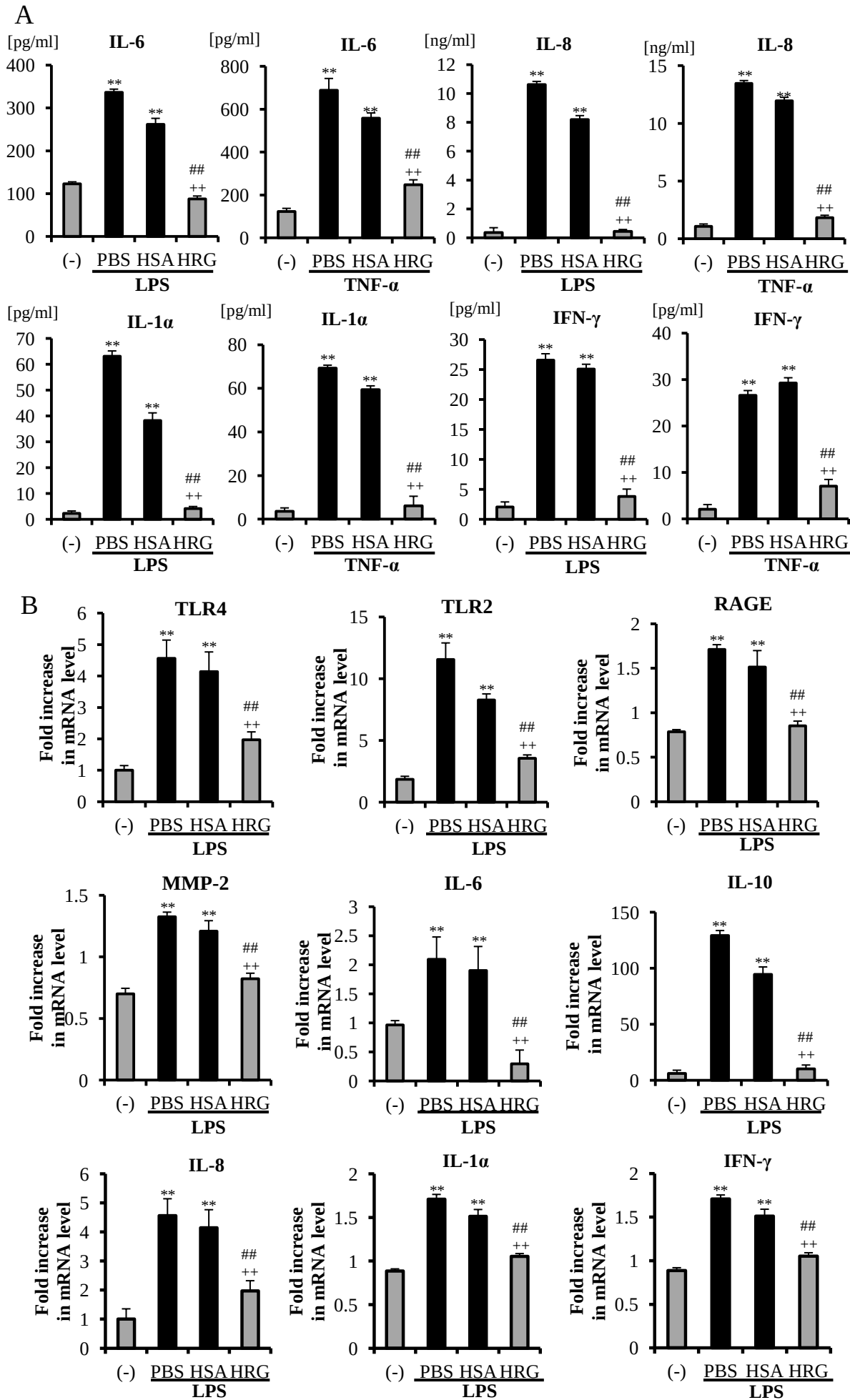


Figure. 5

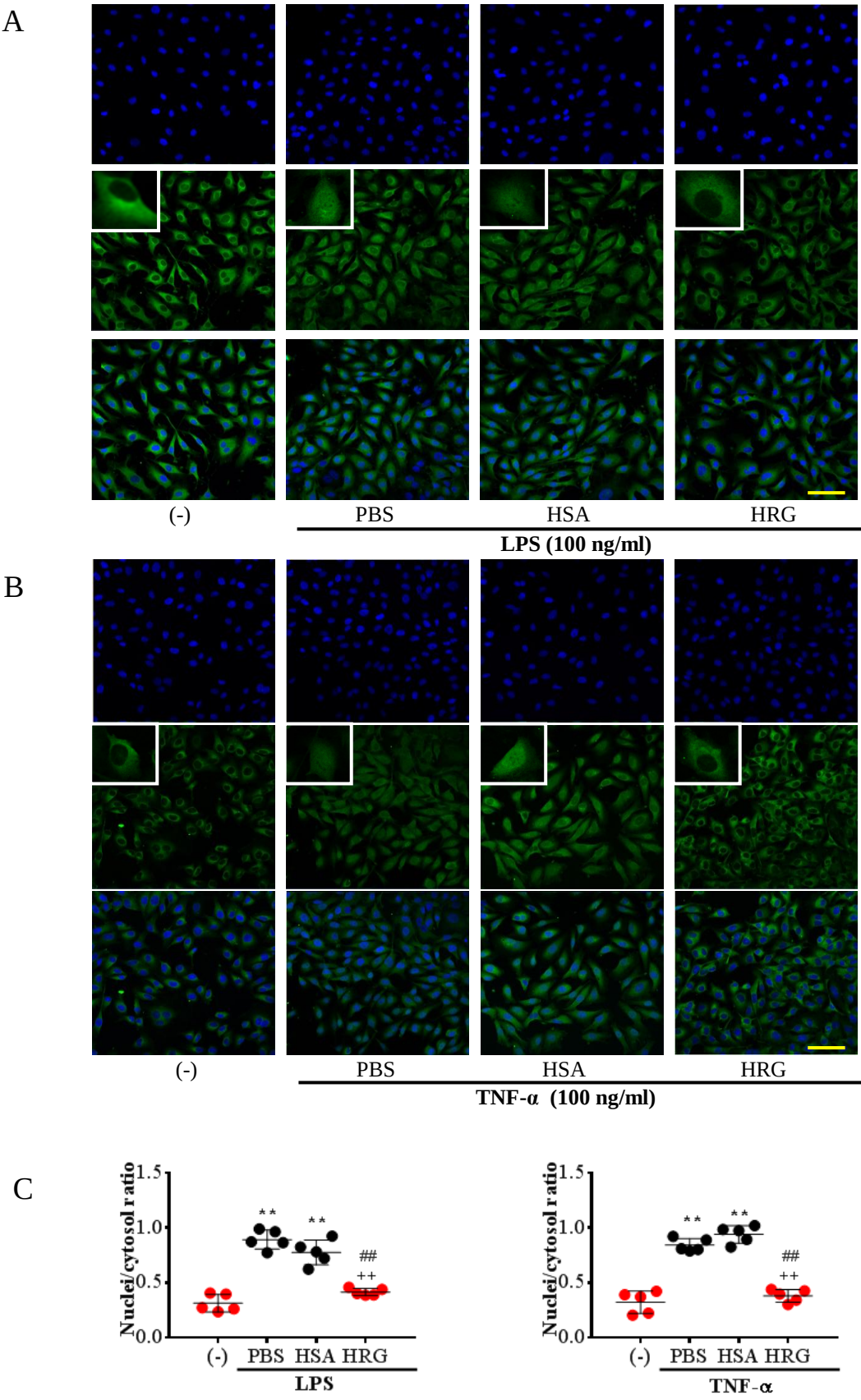


Figure. 6

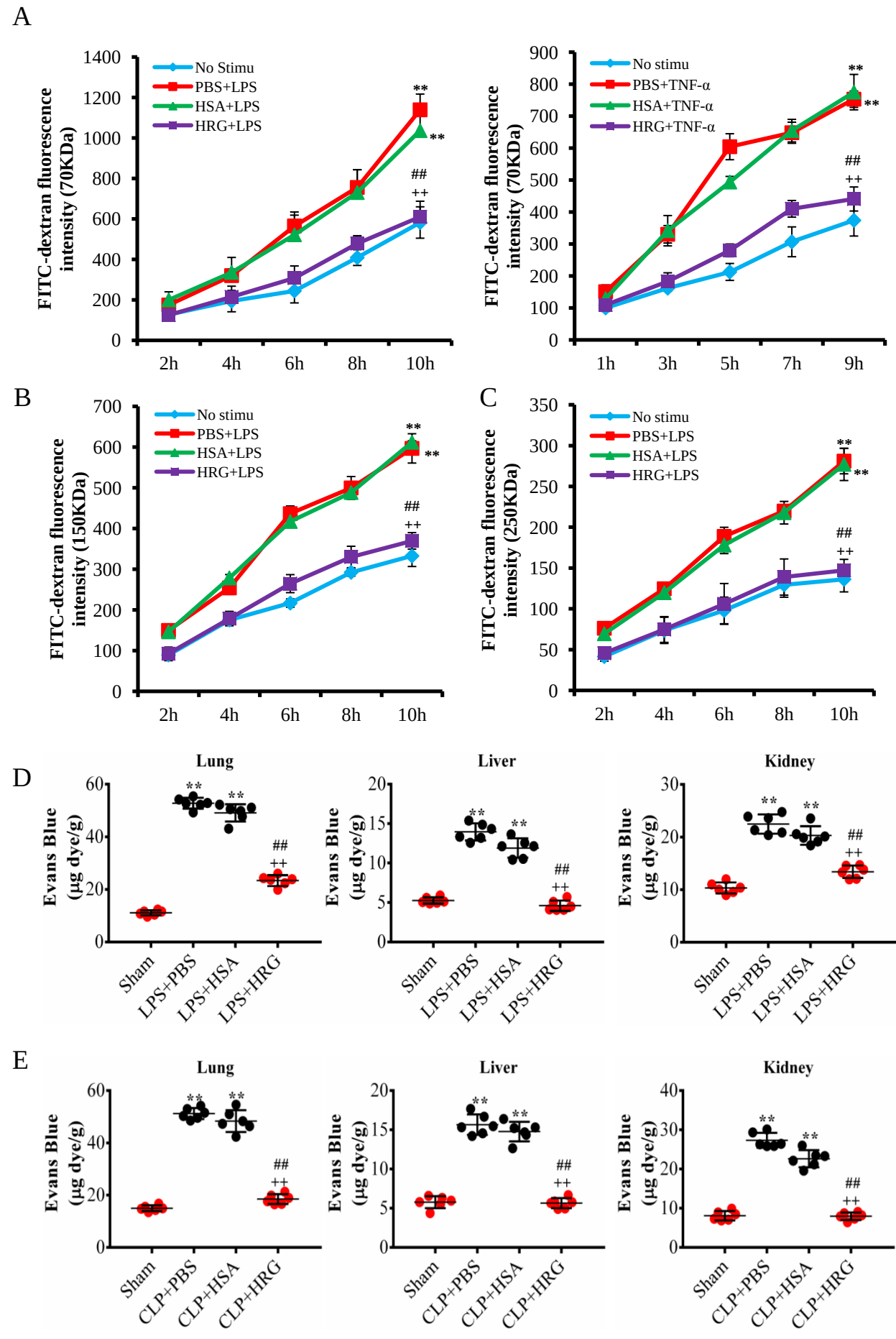
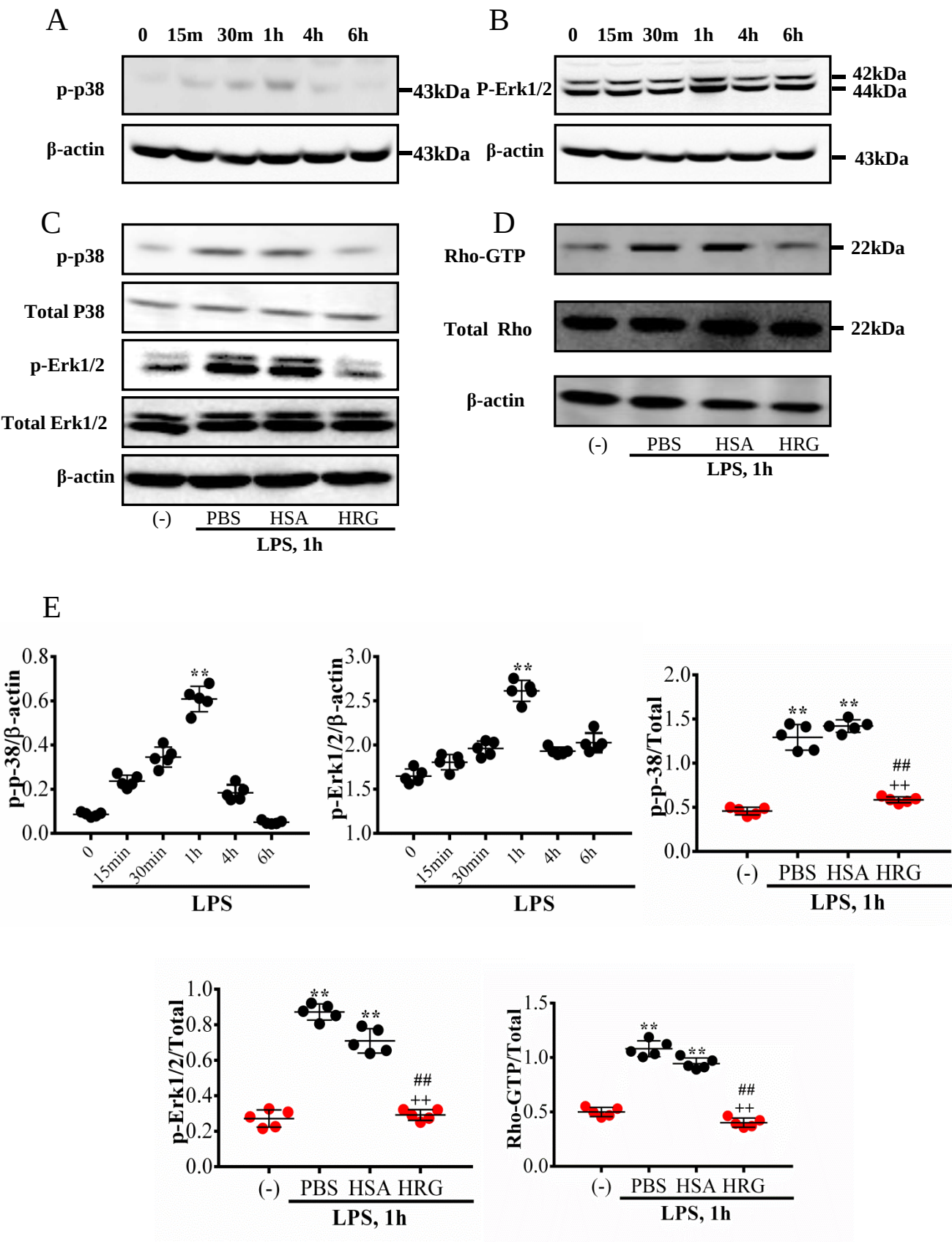


Figure. 7



SUPPLEMENTARY DATA

Histidine-rich glycoprotein ameliorates endothelial barrier dysfunction through regulation of NF- κ B and MAPK signal pathway

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Supplementary Methods

Primary Human Lung Microvascular Endothelial Cells (HMVECs) Culture

Primary human lung microvascular endothelial cells (HMVECs) was obtained from Lonza (#CC-2527). HMVECs were cultured in EBMTM-2 Basal Medium (Lonza CC-3156) with recommended supplements EGMTM-2MV SingleQuots Kit (Lonza CC-4147) in 5% CO₂ at 37 °C. After reaching confluence, the endothelial cells were detached from culture flasks with Accutase[®] 10 ml per 75 cm² at 37 °C for 10 min, washed, and resuspended in culture medium. These cells were passaged every 3–4 days, and all experiments were performed with cells kept in culture between three and ten passages.

Immunostaining Assay

EA.hy 926 endothelial cell or HMVEC suspensions (5×10^5 cells/ml) were cultured in 96-well plates for 16 h until confluent. Wash the monolayer with PBS and pretreated with HRG or HSA (1 μ mol/L) for 30 min before the stimulation with LPS or TNF- α (100 ng/ml) at 37 °C in 5% CO₂ atmosphere. After incubation for indicated period, washing the cell with PBS twice, fixation with 4% paraformaldehyde and then permeabilize with 0.1% Triton X-100 in TBS buffer for 10 min. Endothelial cell was stained with Phalloidin-Alexa 568 or anti-VE-cadherin Ab, anti-NF- κ B Ab for 1 h at 37 °C, followed by Alexa Fluor 488 goat anti-rabbit IgG secondary antibody for 1 h at RT. Nucleus were stained with DAPI for 5 min. The samples were observed using confocal microscopy.

Western blotting

EA.hy 926 endothelial cells cultured in six-well plates were collected with RIPA lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1mM DTT, 20mM β -glycerophosphate and protease/phosphatase inhibitors added immediately before use) and then electrophoresed on polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 10% skim milk for 1 h and incubated overnight at 4 °C with rabbit anti-ZO-1 Ab, anti-Occludin Ab (rabbit, abcam) followed by goat anti-rabbit IgG- HRP (MBL, Nagoya, Japan) for 2 h at RT. The

signals were visualized by the luminal-based enhanced chemiluminescence (ECL) HRP substrate method (Thermo Fisher Scientific, MA). An Image Quant LAS4000 system was used for detection, and images were analyzed with ImageJ software ver. 1.51.

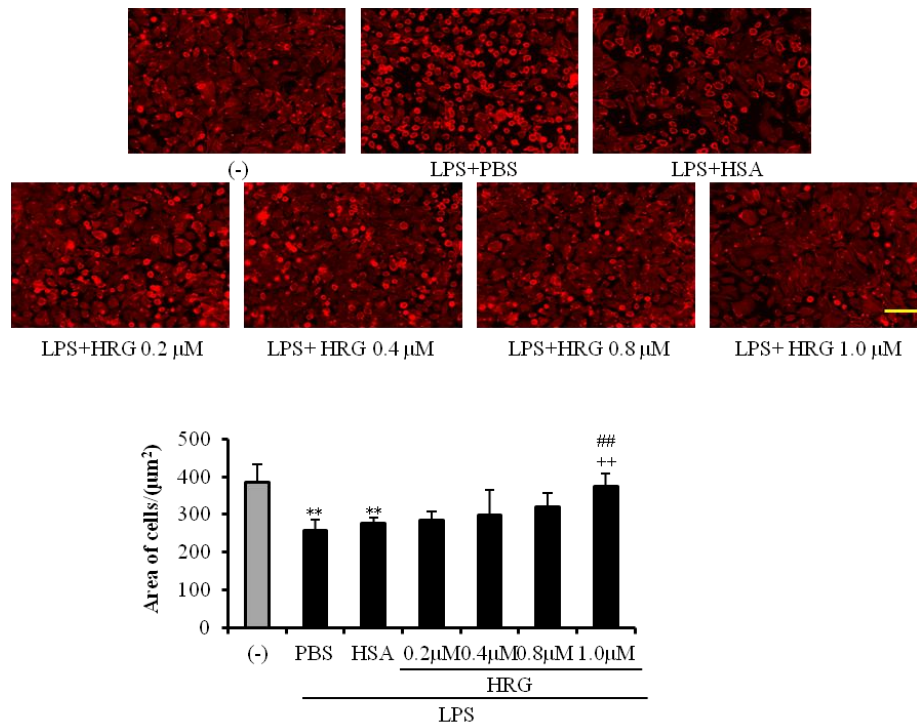
Cytometric Bead Array (CBA)

IL-6, IL-10 and TNF- α concentration in the serum from mouse at 12 h after LPS injection (10 mg/kg) treated with PBS, HSA, and HRG were measured by CBA kits according to the manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ).

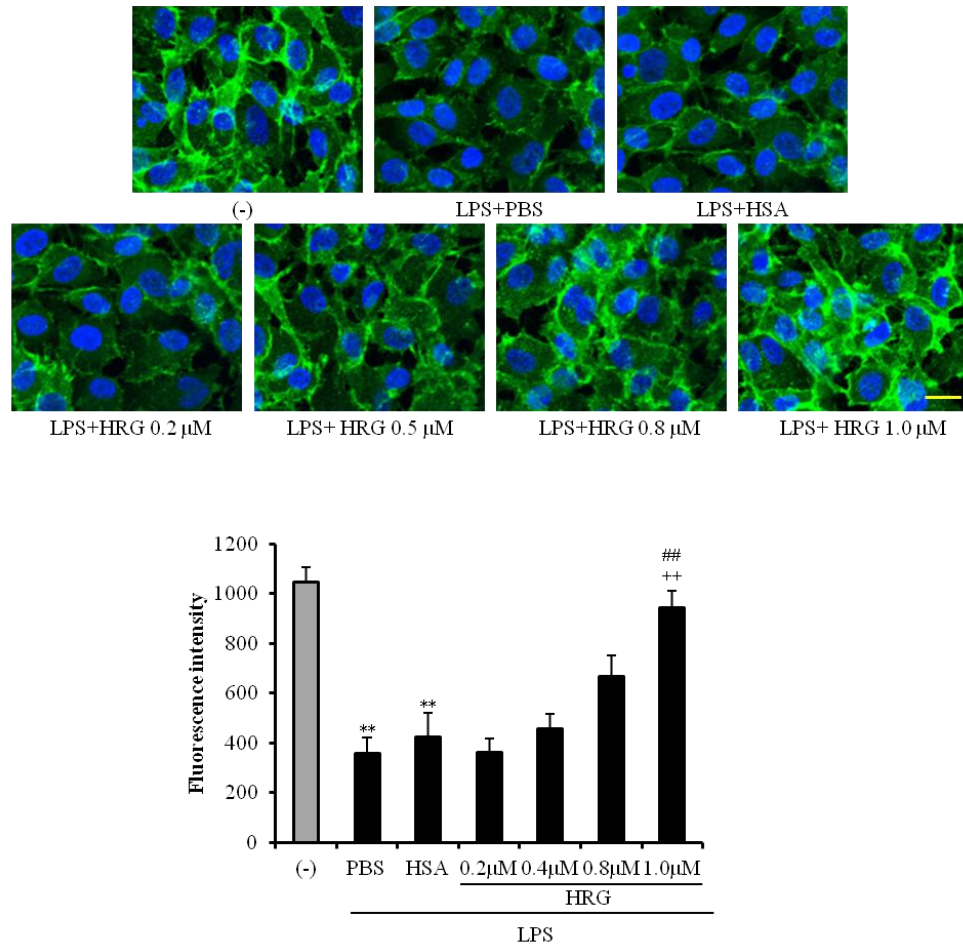
In vitro permeability assay

HMVECs were seeded at a density of 5×10^5 cells/ml onto 24-well plates with 6.5-mm-dia. transwell inserts and a 0.4- μ m pore size polyester membrane (Corning, MA) and cultured until confluent. Cells were pre-incubated for 30 min with HRG/HSA before stimulation with LPS/TNF- α . After 12 h, the culture medium was replaced with medium containing 0.5 mg/ml FITC-dextrane (70 kDa, Sigma-Aldrich) in the upper chamber. The fluorescence in the lower chamber, which represents the permeability of the monolayer of endothelial cells, was detected with the Flex Station 3 (Ex = 492 nm, Em = 518 nm) at different time points.

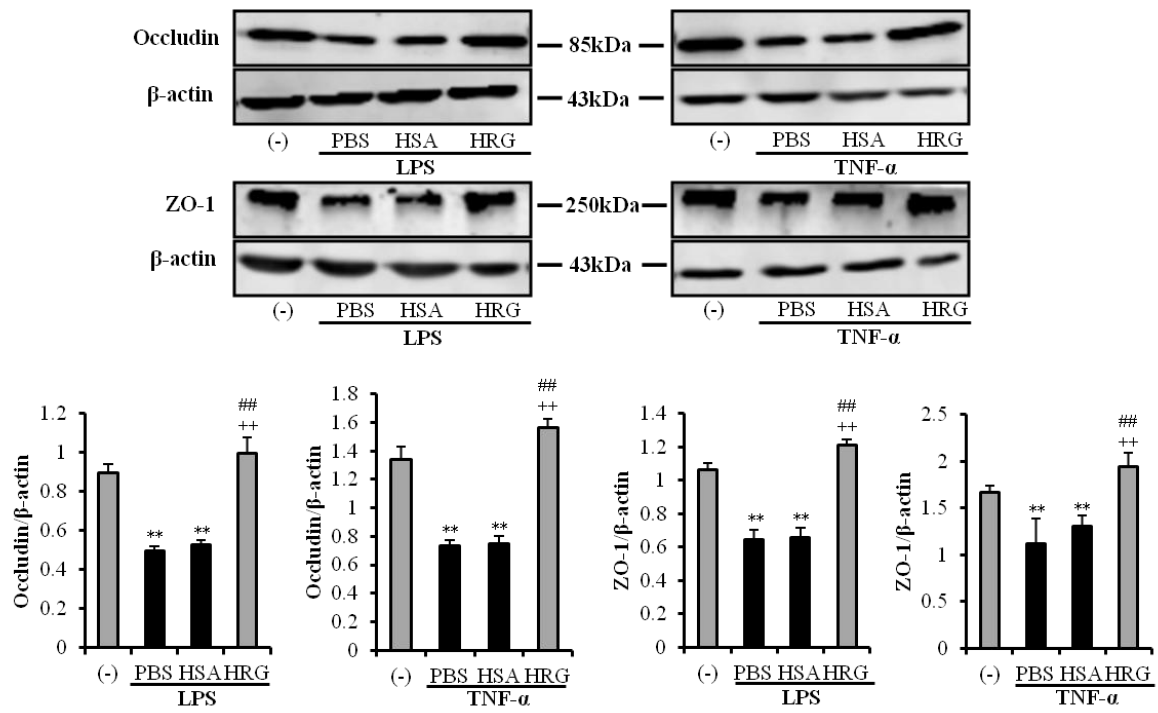
Supplementary Figure



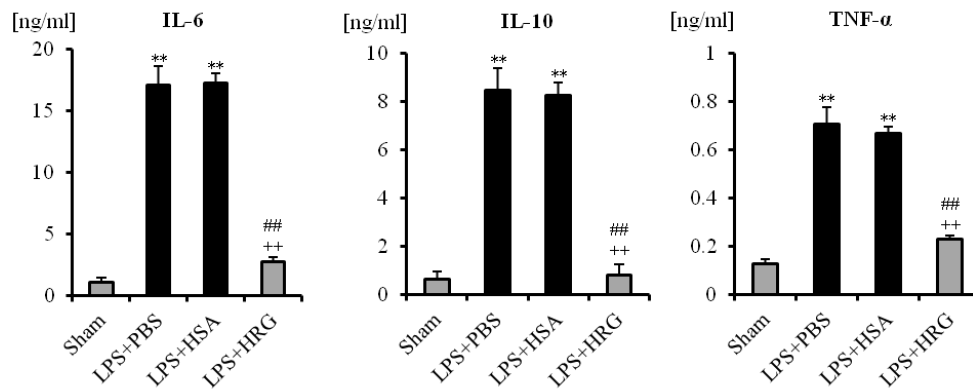
Supplementary Fig. S1. Dose-dependent effects of HRG on the LPS-induced cell morphology changes. EA.hy 926 endothelial cells were cultured on 96-well plates until confluent, and then the cells were incubated with different concentrations of HRG, 1 μ mol/L of HSA or PBS for 30 min at 37°C before stimulation with LPS/TNF- α at 100 ng/ml for 12 h. The actin distribution and morphology of the endothelial cells were analyzed by fluorescence using Alexa Fluor 568-phalloidin. The images are representative of three independent experiments. Scale bar = 20 μ m. Cell areas were quantified using ImageJ software. The results in graphs are the means $\pm$ SEM. One-way ANOVA followed by the *post hoc* Fisher test, (n=5 per group), **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.



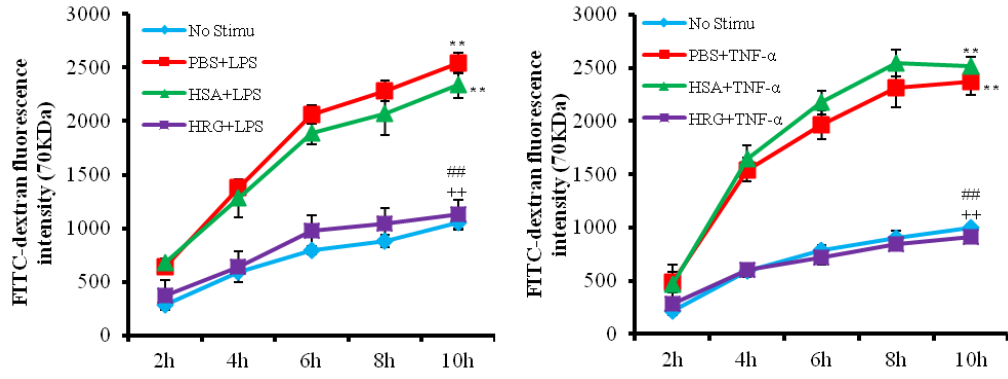
Supplementary Fig. S2. Dose-dependent effects of HRG on the LPS-induced loss of VE-cadherin. EA.hy 926 endothelial cells grown to confluence on 96-well plates were incubated with serum-free medium. The cells were stimulated with 100 ng/ml LPS for 6 h after treatment with different concentrations of HRG or 1 μ mol/L HSA for 30 min. After stimulation, the cells were fixed with 4% paraformaldehyde for 20 min at RT. Cells were incubated with anti-VE-cadherin mAb for 2 h at RT, and then stained with Alexa Fluor 488 (green) goat-anti-rabbit IgG. The pictures are representative from three experiments. Scale bar = 20 μ m. The fluorescence intensity of VE-cadherin in each group was measured with ImageJ software. One-way ANOVA followed by the *post hoc* Fisher test, (n=5 per group), **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.



Supplementary Fig. S3. HRG prevents the loss of intercellular junctions. Quantification results of the junction molecules ZO-1 and occludin of EA.hy 926 endothelial cells by western blotting. Data are mean $\pm$ SEM in the graph (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ⁺⁺p<0.05 vs. PBS and HSA.

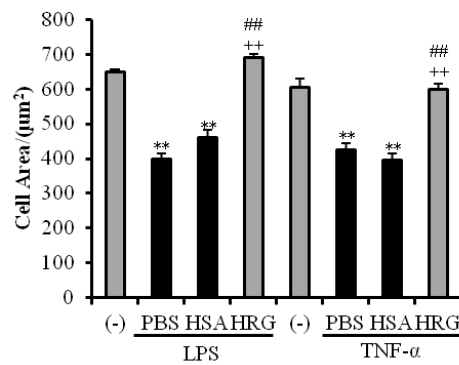
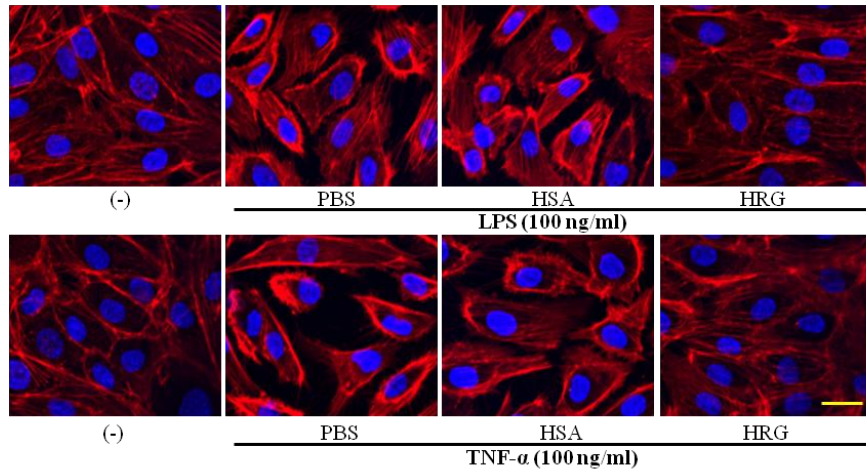


Supplementary Fig. S4. Effects of HRG on the cytokine levels in the serum of LPS-injected mice. Mice were given an intravenous injection of LPS (10 mg/kg) and then treated with HRG or HSA (20 mg/kg) for 12 h. The serum samples for the determination of cytokines were collected 12 h after LPS injection. Data are shown in means $\pm$ SEM (n=6 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. sham, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

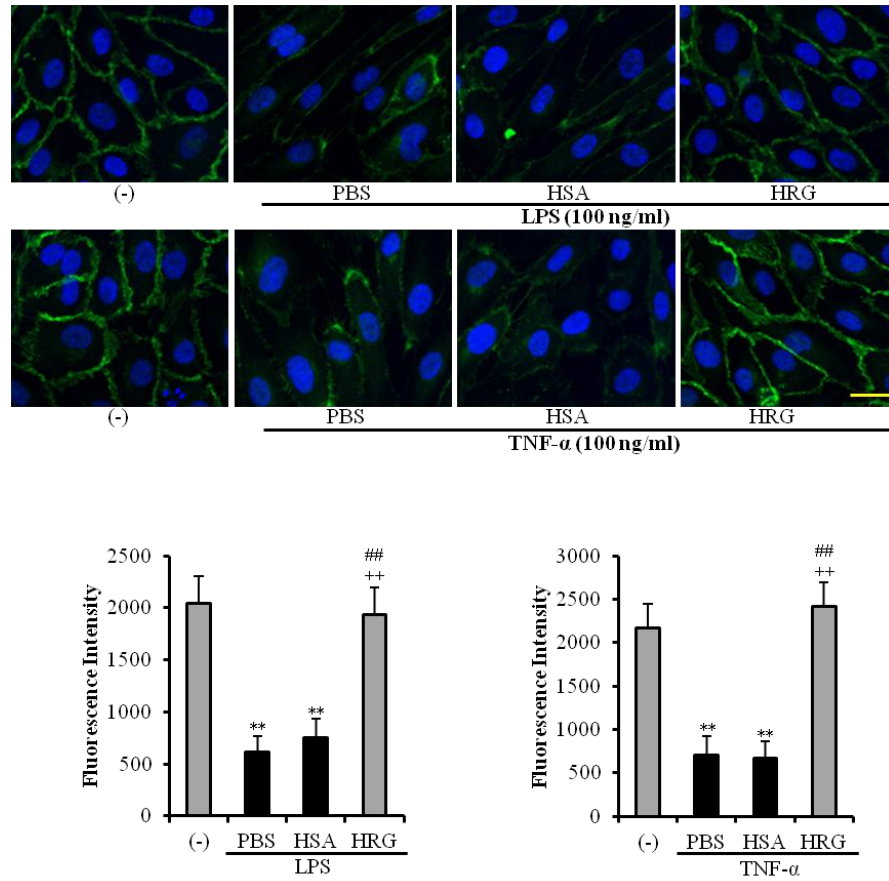


Supplementary Fig. S5. Protection effects of HRG on the HMVECs permeability in vitro.

Effects of HRG on LPS/TNF- α -induced hyperpermeability of the HMVECs monolayer. The graph shows the fluorescence intensity of 70-kDa FITC-dextran leakage from transwell filters to the lower chamber. Data are means $\pm$ SEM in the graph (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

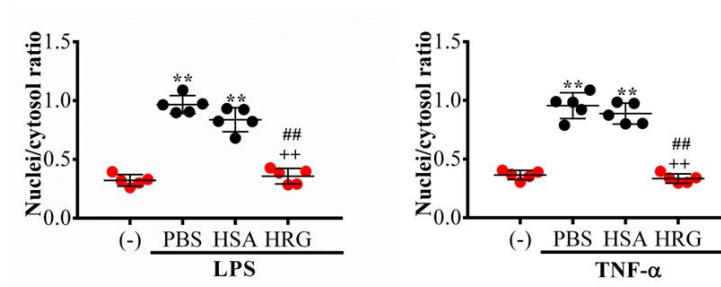
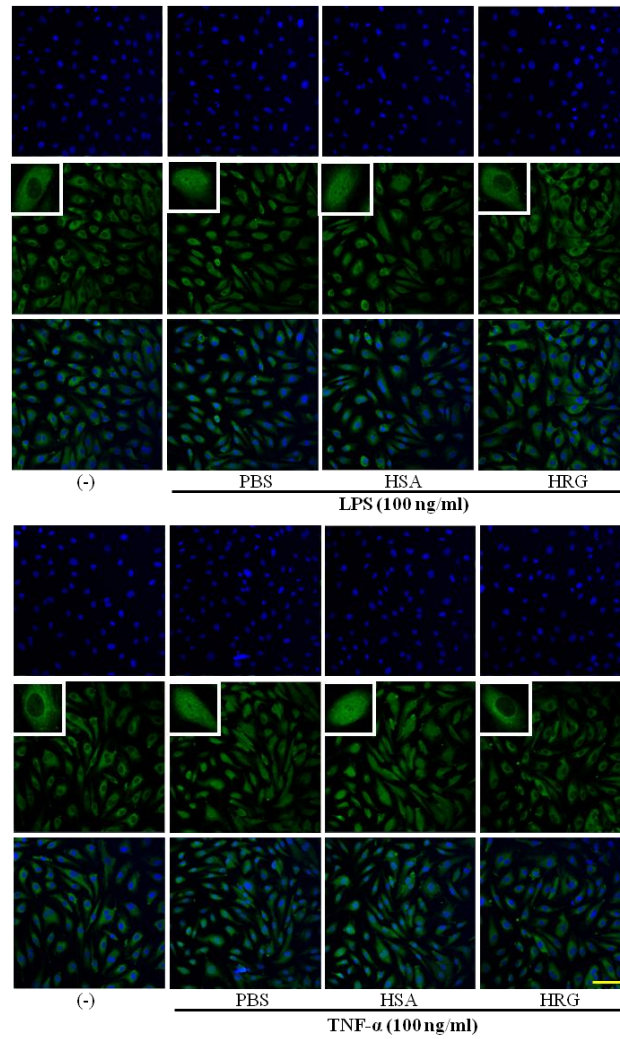


Supplementary Fig. S6. Effects of HRG on LPS/TNF- α -induced cytoskeleton reorganization in HMVECs. HMVECs were incubated with 1 μ mol/L of HSA, HRG, or PBS for 30 min before stimulation with LPS/TNF- α 100 ng/ml for 12 h. The actin distribution and morphology of endothelial cells were analyzed by fluorescence using Alexa 568-phalloidin. Actin staining in red, nucleus staining is blue. Images are representative of three independent experiments. Bar = 20 μ m. Cell areas were quantified using ImageJ software. The results in graphs are the means $\pm$ SEM (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.



Supplementary Fig. S7. HRG prevents the loss of intercellular junction in HMVECs.

Immunostaining results of VE-cadherin in HMVECs stimulated with 100 ng/ml LPS or TNF- α for 6 h after treatment with HRG or HSA. Cells were incubated with anti-VE-cadherin mAb for 2 h and then stained with Alexa Fluor 488 (green) goat-anti-rabbit IgG. The cells were also stained with DAPI (blue) to visualize the nuclei. The pictures are representative from three independent experiments. Scale bar = 20 μ m. One-way ANOVA followed by the *post hoc* Fisher test, (n=5 per group), **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.



Supplementary Fig. S8. HRG suppresses the LPS/TNF- α -induced NF- κ B activation in HMVECs. Immunostaining results of NF- κ B in endothelial cells stimulated with 100 ng/ml LPS for 12 h or 100 ng/ml TNF- α for 6 h after treatment with HRG or HSA. Cells were stained with anti-NF- κ B/p65 mAb for 2 h and then stained with Alexa Fluor 488 (green) goat-anti-rabbit IgG. The results shown are representative of three experiments. Scale bar = 10 μ m. The nuclei/cytosol fluorescence intensity ratio represents the translocation of NF- κ B from the cytoplasm to nucleus. Data are means $\pm$ SEM in the graph (n=5 per group). One-way ANOVA followed by the *post hoc* Fisher test, **p<0.05 vs. control, ##p<0.05 and ++p<0.05 vs. PBS and HSA.

Supplementary Table

RT-PCR Primer Sequence

mRNA	Sense primer	Anti-sense primer
MMP2	5'-CATTCCGCTTCCAGGGCACAT-3'	5'-GCACCTTCTGAGTTCACCAA-3'
TLR4	5'-ACTCCCTCCAGGTTCTTGATTAC-3'	5'-CGGGAATAAAGTCTCTGTAGTGA-3'
IL-6	5'-GAACTCCTTCTCCACAAGCGCCTT-3'	5'-CAAAAGACCAGTGATGATTTTCACCAGG-3'
TNF- α	5'-GAGTGACAAGCCTGTAGC-3'	5'-CCCTTCTCCAGCTGGAAG-3'
β -actin	5'-AGCGGGAAATCGTGCGTG-3'	5'-CAGGGTACATGGTGGTGCC-3'
IL-10	5'-CTGAGAACCAAGACCCAGACATCAAGG-3'	5'-GTCAGCTATCCCAGAGCCCCAGATCCG-3'
TLR-2	5'-GCCAAAGTCTTGATTGATTGG-3'	5'-TTGAAGTTCTCCAGCTCCTG-3'
RAGE	5'-GCCCTCCAGTACTACTCTCG-3'	5'-TGTGTGGCCACCCATTCCAG-3'
IL-8	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	5'-TCTCAGCCCTCTTCAAAAACCTTCTC-3'
IL-1 α	5'-GAATGACgCCCTCAATCAAAGT-3'	5'-TCATCTTGGGCAGTCACATACA-3'
IFN- γ	5'-TGACTTGAATGTCCAACGCAAAG-3'	5'-GCAGGACAACCATTACTGGGATG-3'