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Barrier Protective Effects of 2,4,6-Trihydroxy-3-Geranyl Acetophenone on Lipopolysaccharides-Stimulated Inflammatory Responses in Human Umbilical Vein Endothelial Cells

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Abstract

Pharmocological relevance:

2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), is a phloroglucinol compound found naturally in *Melicope ptelefolia. Melicope ptelefolia* has been used traditionally for centuries as natural remedy for wound infections and inflammatory diseases.

Aim of the study:

Endothelial barrier dysfunction is a pathological hallmark of many diseases and can be caused by lipopolysaccharides (LPS) stimulation. Therefore, this study aims to investigate the possible barrier protective effects of tHGA upon LPS-stimulated inflammatory responses in human umbilical vein endothelial cells (HUVECs).

Materials and methods:

HUVECs were pretreated with tHGA prior to LPS stimulation, where inflammatory parameters including permeability, monocyte adhesion and migration, and release of pro-inflammatory mediators were examined. Additionally, the effect of tHGA on F-actin rearrangement and adhesion protein expression of LPS-stimulated HUVECs was evaluated.

Results:

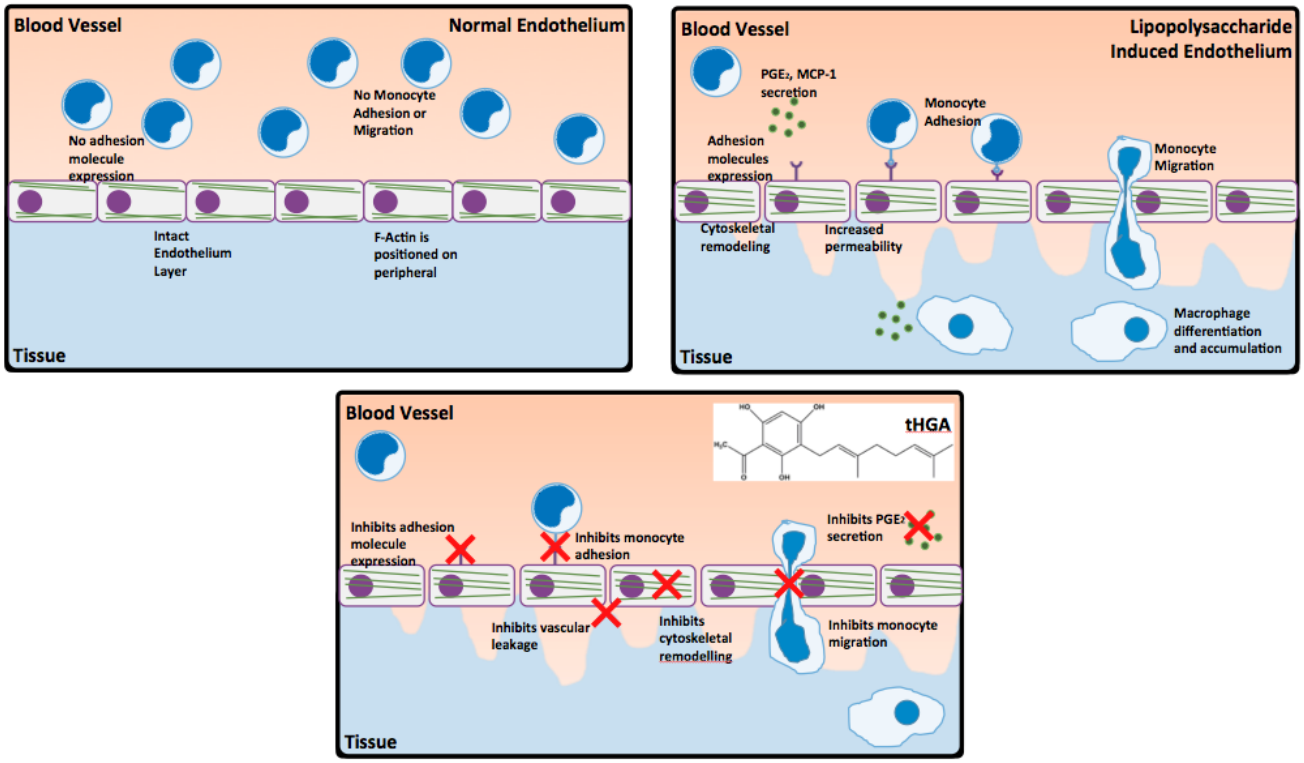
It was found that pretreatment with tHGA inhibited monocyte adhesion and transendothelial migration, reduced endothelial hyperpermeability and secretion of prostaglandin E_2 (PGE_2). Additionally, tHGA inhibited cytoskeletal rearrangement and adhesion protein expression on LPS-stimulated HUVECs.

Conclusion: As the regulation of endothelial barrier dysfunction can be one of the therapeutic strategies to improve the outcome of inflammation, tHGA may be able to preserve vascular barrier integrity of endothelial cells following LPS-stimulated dysfunction, thereby endorsing its potential usefulness in vascular inflammatory diseases.

Keywords

2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), *Melicope ptelefolia*, phloroglucinol, barrier protective effect, endothelial dysfunction, lipopolysaccharides.

Graphical Abstract



1. Introduction

The endothelium is a functional barrier that lines the blood vessel, in which this barrier plays major roles in homeostasis including coagulation, fibrinolysis and immune responses. During inflammation, the endothelium is activated and releases a cascade of inflammatory mediators such as MCP-1 and IL-8 for recruitment of monocytes and neutrophils (Gavard, 2009; Zhang et al., 2011). Additionally, the endothelium also upregulates the expression of adhesion molecules and dissociates the tight junctional proteins to facilitate diapedesis and fluid permeation (Gavard, 2009; Harmey et al., 2002). In between the alteration of protein expression, the endothelium permeability is increased, and the recruitment of circulating leukocytes is heightened. Therefore this initiates the vascular barrier

dysfunction cascade (Thiermermann et al., 1995). This is deleterious to the host as such hyperinflammatory activity may cause systemic shock and tissue damage, both of which may be fatal. Therefore, intervention on such severe inflammatory state is necessary.

LPS is found on the membrane of gram-negative bacteria, and it is frequently found in high concentration in patients who are suffering from septicemia and endotoxemia (Rivers et al., 2001). Activation of TLR4 by LPS will lead to downstream upregulation of various pro-inflammatory signaling molecules that play important role in propagating inflammatory responses such as NF-κB (Hoefen & Berk, 2002) and MAP kinases (Sawa et al., 2008). In high concentrations, LPS may lead to fatal conditions such as multiple organ dysfunction and septic shock (Dellinger et al., 2004), both of which are linked to vascular barrier disruption (Angus & Van der Poll, 2013; Balk, 2000). It has been suggested that therapeutic approaches aimed at maintaining vascular barrier integrity constitute a potential approach in the management of sepsis (Dellinger et al., 2004; Goldenberg et al., 2011).

Melicope ptelefolia (Champ. ex Benth.) is a shrub growing in many areas of the Southeast Asia commonly used as a traditional Chinese medicine for the treatment of inflammatory reactions including abscesses, wound infection, eczema and dermatitis (Jiangsu New Medical School, 1986). In Malaysia, the shoots and young leaves of *Melicope ptelefolia* are believed to be high in nutritional and medicinal values and amongst the popular ‘ulam’ or green salad favored by the local Malay population. Our research group had successfully isolated a new natural product namely 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA) from *Melicope ptelefolia* (Shaari et al., 2006). tHGA was identified as one of the three marker compounds that are found abundantly in young leaves but not in mature leaves of *Melicope ptelefolia* (Shuib et al., 2011). tHGA is a drug-like compound containing a phloroglucinol structural-core as the bioactive principle (Ng et al., 2006; Shaari et al., 2006) (Figure 1).

Our research group previously reported significant antioxidant and nitric oxide inhibitory activities of *Melicope ptelefolia,* suggesting its potential anti-inflammatory properties (Abas et al., 2006). Our recent work also demonstrated that tHGA prevents airway inflammation in an ovalbumin-induced murine model of acute allergic asthma. The airway hyperresponsiveness, pulmonary cellular infiltration, goblet cell metaplasia, cytokine and cysteinyl leukotriene secretion and systemic IgE concentrations were all reduced following systemic treatment of tHGA (Ismail et al., 2012). Bioassay studies have shown that tHGA exerts dose-dependent inhibition against 5-lipoxygenase (5-LOX) and both cyclooxygenase (COX) isoforms (Shaari et al., 2011), both of which play an important role in mediating LPS-stimulated inflammation. Virdis et al, revealed that inhibition of COX-2 improved the outcome of LPS-stimulated endothelial dysfunction due to reduction of pro-inflammatory signal transduction and reactive oxygen species (Virdis et al., 2005), therefore giving insights on the potential of tHGA on inhibiting LPS-stimulated vascular dysfunction. There have not been any attempts to evaluate the use of tHGA as an adjunct therapeutic in sepsis or LPS-associated endothelial dysfunction. Hence, this study was carried to evaluate the barrier protective effect of tHGA upon LPS-stimulated endothelial dysfunction.

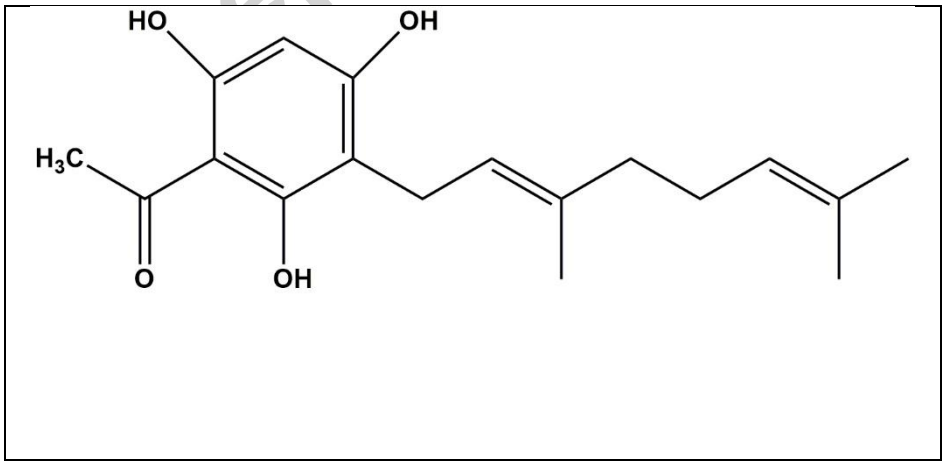


Figure 1: Structure of 2,4,6-Trihydroxy-3-Geranyl Acetophenone

2. Materials and Methods

2.1 Reagents

Lipopolysaccharide (O111:B5), Evans Blue, Albumin and BCECF-AM were purchased from Sigma (St Louis, MO, USA). Sodium Fluorescein (Na-F), dimethyl sulfoxide and 3-(4,5-Dimethylthiazol-2-yl)-2-Diphenyltetrazolium Bromide (MTT) were purchased from Amresco (Solon, OH, USA). Water soluble tetrazolium-1 (WST-1) was purchased from Millipore (Billerica, MA, USA). Cell culture inserts and Collagen I was purchased from BD Biosciences (Franklin Lake, NJ, USA). Alexa Fluor-488 conjugated phalloidin was purchased from Invitrogen (Carlsbad, CA, USA). Mouse Anti-Human ICAM-1 (sc107), Mouse Anti-Human VCAM-1 (sc-13160) and goat anti-mouse IgG (sc-2005) was purchased fron Santa Cruz Biotechnology (Santa Cruz, CA)

2.2 Synthesis of tHGA

tHGA was synthesized according to previously described method (Ismail et al., 2012). A well stirred mixture of phloracetophenone (1.000 g, 6 mmol), geranyl bromide (0.876 g, 4.80 mmol), and anhydrous potassium carbonate (0.415 g, 3.00 mmol) in dry acetone (3.5 mL) was refluxed for 6 h. The reaction mixture was filtered and evaporated under reduced pressure to give an oily orange residue that was purified by flash column chromatography on Si gel (petroleum ether-EtOAc, 10:1) to afford 2,4,6-Trihydroxy-3-geranylacetophenone (tHGA) as a light yellow powder; mp 128–130 °C. ^{1}H NMR (CD_3OD) δ_H1.58 (3 H, s, Me), 1.63 (3 H, s, Me), 1.76 (3 H, s, Me), 2.64, (3H, s, COMe), 1.96 (2H, q, J = 7.5 Hz), 2.06 (2H, m), 3.21 (2H, d, J = 6.5 Hz), 5.08 (1H, t, J = 7 Hz), 5.20 (1H, t, J = 6.5 Hz), 5.92 (1H, s, ArH); IR (KBr) ν_{max} 3405, 1627 cm-1; EIMS m/z (%) [M] + 304 (38), 289 (3), 261 (9), 235 (25), 181 (100).

2.3 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Cascade Biologics. The cells were maintained in Endogro-LS Complete Media Kit (Merck Milipore) at 37^{o}C in a humidified incubator with 5% CO_2. U937, a human monocytic cell line, was purchased from ATCC. The cells were maintained at a density of 2×10^5 - 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FBS. For this study, only HUVECs between passage 3 to passage 5 were used.

2.4 MTT Cell Viability Assay

Effect of tHGA on viability of cells were examined using MTT reagent. HUVECs were grown at a concentration of 4×10^3 HUVECs/well. The seeding concentration is determined based on MTT optimization assay whereby the optimal seeding range of HUVECs for MTT is between 1.25×10^3 to 5×10^4 cells/well in 96 well plate (data not shown). On the next day, different concentrations of tHGA with the presence of 1 µg/mL LPS were added into each individual well and co-incubated in a humidified CO_2 incubator for 24 h at 37°C. MTT (5 mg/mL) was then added followed by incubation in CO_2 incubator at 37^{o}C of 4 h. Finally, live cells containing formazon salt were then lysed with DMSO, and the amount of formazon salt was determined via measuring OD at 570nm using microplate reader (Tecan Austria GmbH, Austria).

2.5 Adhesion Assay

Monocyte adhesion to HUVECs was performed as described previously with some modification (Tham et al., 2015). Confluent HUVECs in 96 well plates were first pretreated with tHGA or Dexamethasone for 6 h, followed by LPS stimulation for 5 h. Meanwhile, U937 cells were prelabelled with 4 µg/mL BCECF-AM for 1 h immediately prior to co-culture. Following stimulation, HUVECs were washed with PBS, followed by co-cultured with 1×10^5 U937 in 100 µL media for 1 h to allow adhesion of the monocytes to the endothelial monolayer. Non-adherent cells were then washed thrice with PBS, and

adhered U937 were lysed with 0.2% Triton-X 100. The fluorescence intensity was quantified at 485-nm excitation and 530-nm emission using a spectrofluorometer (Tecan M200 Infinite, Mannedorf, Switzerland).

2.6 Cell Migration Assay

Cell migration assay was performed using 8 µm PET cell culture inserts. The inserts allow formation of an inner and outer chamber, where migration of monocytes across the chambers will be measured. Following coating of the inner chamber of the insert with 1 µg/cm^2 collagen, HUVECs were seeded until confluent on the insert membrane. On the day of the experiment, HUVECs were pretreated with tHGA for 6 hours and stimulated with LPS for 4 h. The insert was then washed and transferred to a clean well containing DMEM with 20% FBS. Serum starved U937 (1x10^6 cells/well) were added on the upper chamber and allowed to migrate through the HUVEC monolayer for 4 h. Migrated cells on the bottom chamber were harvested and incubated with WST-1 dye for 4 h before measuring the absorbance at 450-nm using a spectrophotometer (Tecan M200 Infinite, Mannedorf, Switzerland).

2.7 Permeability Assay

Permeability assay was performed using a modified protocol from previous studies using Sodium Fluorescein (Na-F) and Evans Blue Albumin (EBA) (Takata et al., 2013; Yamada et al., 2014). HUVECs were seeded until confluent on the membrane of the insert. Upon pretreatment with tHGA for 6 h and stimulation with LPS for 4 h, the insert was washed and transferred to a clean well containing 500 µL assay buffer (136mM NaCl, 0.9mM $CaCl_2$, 0.5mM $MgCl_2$, 2.7mM KCl, 1.5mM KH_2PO_4, 10mM NaH_2PO_4, 25mM glucose and 10mM HEPES, pH7.4). Then, 200 µL of assay buffer containing 10µg/mL Na-F and 165µg/mL EBA was added into the insert. Both Na-F and EBA were allowed to permeate for 10 min. The lower chamber buffer was collected after 10 min, and permeated Na-F

(Fluorescence intensity: 485-nm excitation, 535-nm emission) and EBA (Optical Density: 620-nm) were measured using dual spectrophotometer/spectrofluorometer (Tecan M200 Infinite, Mannedorf, Switzerland). The permeability index was normalized according to a previously described method (Maruo et al., 1992).

2.8 F-Actin Staining

Phalloidin is a compound isolated from *Amanita phalloides* that binds specifically to F-actin. Phalloidin conjugated with Alexa-Fluor 488 was used for staining of F-actin. HUVECs were first seeded in 4-well chamber slides. Then, the cells were pretreated with tHGA or Dexamethasone for 6 h, followed by stimulation with LPS for 24 h. The cells were then washed with PBS, and fixed using 4% paraformaldehyde for 10 mins. After two washes with PBS, the cells were permeated with 0.2% Triton X-100 for 5 mins. Then the cells were blocked with 1% BSA for 20 mins to reduce nonspecific binding. The cells were then stained using Alexa-Fluor 488 phalloidin diluted in 1% BSA (1:200) for 20 mins and counterstained with 1 µg/mL Hoescht 33342. Finally, the slides were mounted using MOWIOL 4-88 Mounting Agent. F-Actin fibers were then examined using Leica DM2500 Microscope (IL, USA) under 400x magnification.

2.9 Western Blot Analysis

Confluent HUVECs were pretreated with tHGA for 6 h prior to induction with LPS stimulation for 24 h. The cells were lysed in RIPA lysis buffer (150mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50mM Tris, pH8.0) to obtain whole cell lysate. The protein amount in whole cell lysate was quantified by using BCA assay. Equal amount of protein (20 µg) was electrophoresed on 8% SDS-polyacrylamide gel prior to blotting into PVDF membrane using Mini Trans-Blot Cell (Biorad, CA). The membrane was then blocked with 5% BSA in Tris-Buffered Saline with 0.05% Tween-20 (TBST)

before overnight incubation with polyclonal antibody specific for ICAM-1 (1:1000), VCAM-1 (1:1000) or β-Actin (1:10000). After washing, the membrane was hybridized with HRP-conjugated antibody specific to the primary antibody (1: 2000). The membrane was incubated with Western Bright Sirius HRP Substrate (Advansta, CA) and viewed under chemiluminescence by a CCD camera imaging system (Vilber Lourmet, Marne-la-Vallee, France). Band intensity was quantified by Image J software and normalized to β-Actin.

2.10 ELISA for MCP-1 and PGE_2

The secretion of MCP-1 (R&D Systems, Catalog no: DCP00) and PGE_2 (R&D Systems, KGE004B) was determined by enzyme immunoassay according to the manufacturer's protocol (Minneapolis, MN, USA). Briefly, confluent HUVECs in 96 well plate were pretreated with tHGA or Dexamethasone for 6 h, followed by induction with LPS for 24 h in 100 µL of complete media. The cell culture supernatant were collected upon completion of LPS stimualtion for analysis using the ELISA kit, based on the manufacturer's protocol.

2.11 Statistical Analysis

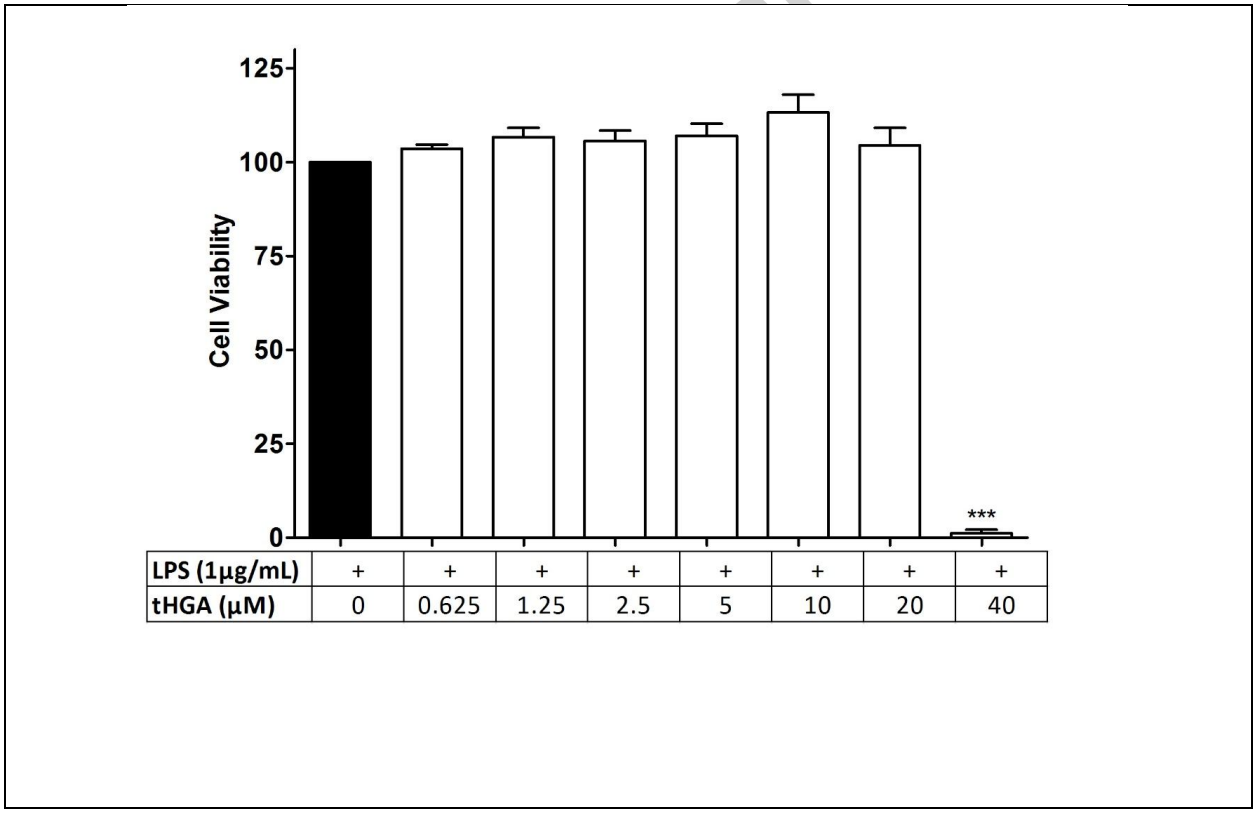
All statistical data are expressed as means ± S.E.M of three independent experiments in triplicate. All analyses were performed using SPSS 22.0. One-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's test were used for all statistical analysis. Values of $P \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. tHGA does not affect HUVECs viability

The results from MTT assay as per summarized in Figure 2 was used to determine the concentrations of tHGA which are non-cytotoxic to HUVECs. The results indicated that tHGA in presence or absence (data not shown) of LPS is non-cytotoxic at concentrations of ≤20 μM. Subsequently, assays were performed using concentrations of 20 μM, 5 μM and 1.25 μM. Dexamethasone (10 μM) or Quercetin (100 μM) were used as drug controls.

Figure 2: tHGA is non-cytotoxic at 20μM and below. HUVECs were seeded at 96-well plate overnight before treatment with LPS and tHGA. tHGA is non-cytotoxic at 20 μM and below, and is cytotoxic at 40 μM and above. Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *** $P \leq 0.001$ significantly different from LPS-stimulated control without any treatment.

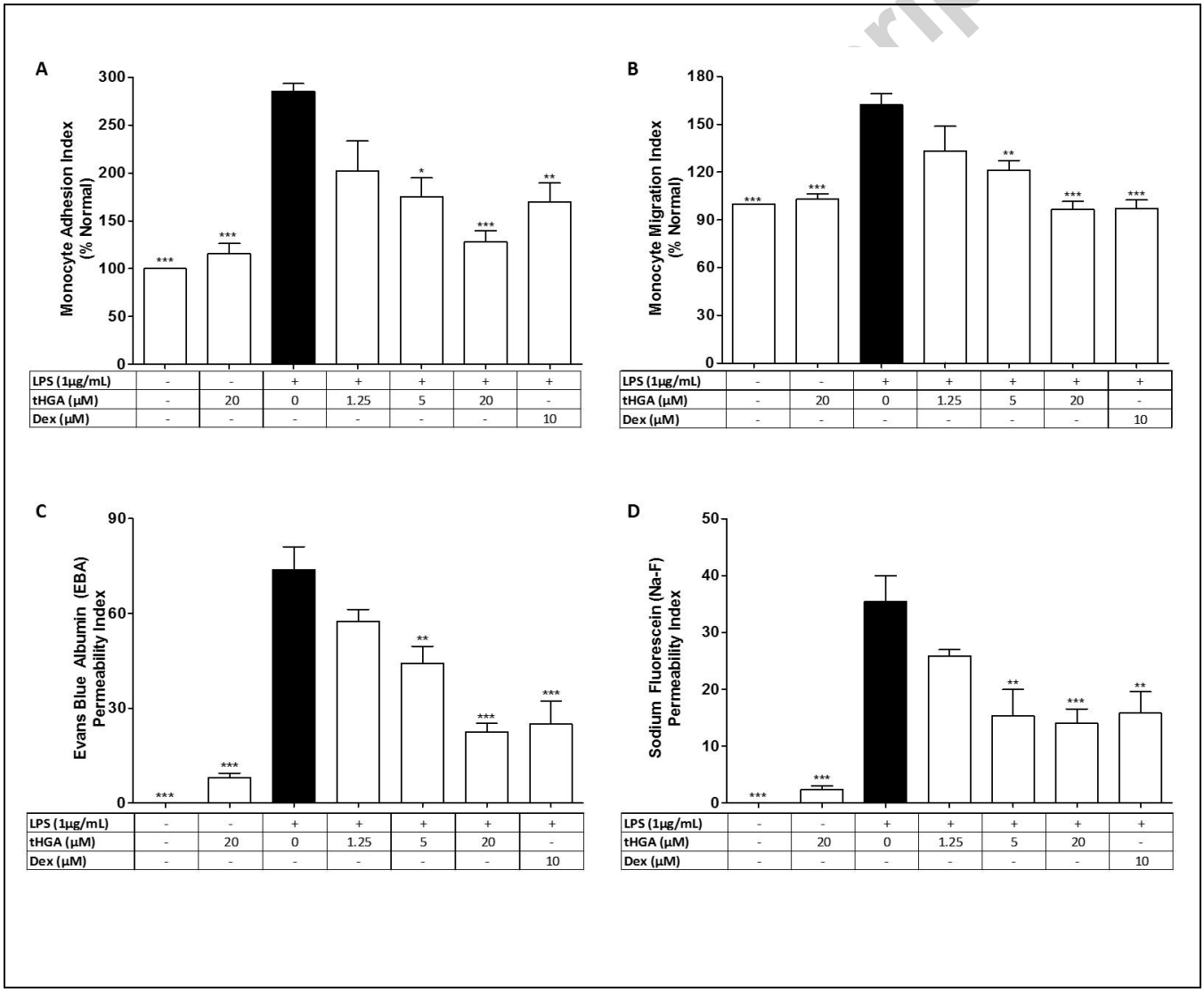


3.2 tHGA reduces monocyte adhesion and transendothelial migration

LPS is known to stimulate the expression of adhesion molecules on endothelial cells to enhance the adhesion and transendothelial migration of leukocytes. We have examined the effect of tHGA on the adhesion of U937 monocytes to HUVECs and subsequent migration through the HUVEC monolayer. Figure 3A shows that upon LPS stimulation, the adhesion of U937 on HUVECs is significantly increased in contrast to unstimulated cells. Pretreatment with tHGA or Dexamethasone caused significant decrease in fluorescence intensity, indicating a lowered adhesion of BCECF-AM labelled U937 to the HUVECs.

Additionally, we examined the capability of tHGA in inhibiting migration of monocytes across activated endothelial cells. HUVECs were first pretreated with tHGA or Dexamethasone prior to LPS stimulation. Figure 3B shows increased monocyte migration following LPS stimulation and 20 µM and 5 µM tHGA effectively inhibited migration of U937 cells across the HUVEC monolayer.

Figure 3: tHGA attenuates barrier dysfunction of LPS-stimulated HUVECs. tHGA significantly reduced monocyte adhesion (A) and transendothelial migration (B) of and through LPS-stimulated HUVEC monolayer at 5 and 20 µM. Additionally, tHGA also significantly reduced leakage of large and small molecules through LPS-stimulated HUVECs, at 5 and 20 µM, as per indicated by Evans Blue Albumin leakage (C) and Sodium Fluorescein leakage (D). Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ significantly different from LPS-stimulated control without any treatment.



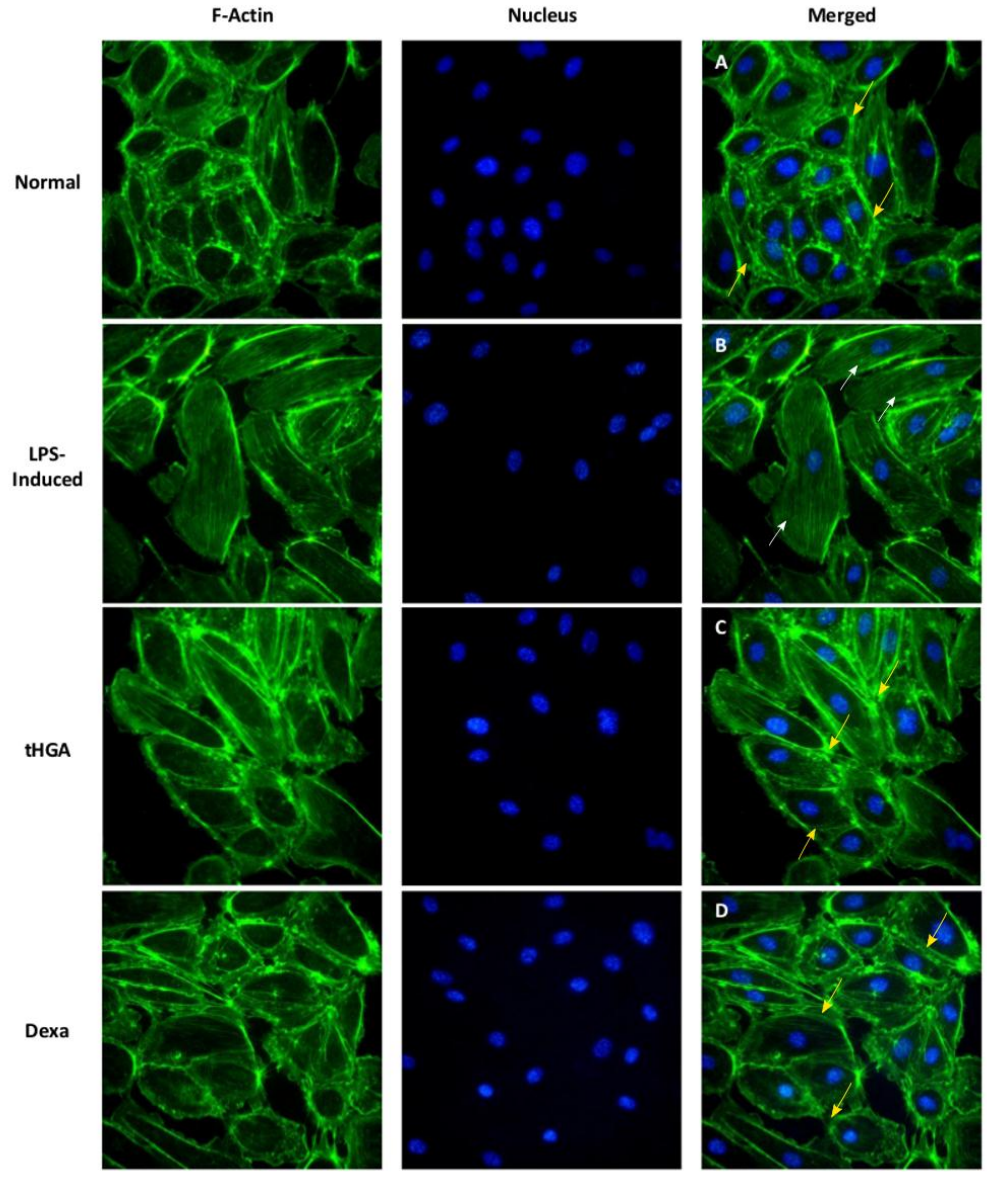
3.3 tHGA reduces permeability of LPS-stimulated HUVEC monolayer

Stimulation of endothelial cells with LPS disrupts monolayer integrity. Therefore it paved ways for blood content, such as proteins, to infiltrate to surrounding tissues (Thiermermann et al., 1995). We examined the effect of tHGA in mediating endothelial monolayer permeability by examining flux of EBA and Na-F across the monolayer. There are two mechanisms for solute permeation through the endothelium: transcellular for large molecules, and paracellular for small molecules (<30μm). In order to mimic both mechanisms of permeation, EBA (MW=67kDa) and Na-F (MW=376 Da) were used to trace permeation via transcellular and paracellular routes, respectively (Maruo et al., 1992). Figures 3C and 3D show that LPS is able to increase the permeability EBA and Na-F, and tHGA is able to suppress the permeability of both tracers at 20 μM and 5 μM. tHGA at 1.25 μM exhibited no significant effect in inhibiting influx of both tracers. This indicated that tHGA may reduce both transcellular and paracellular permeation.

3.4 tHGA preserves F-actin arrangement of LPS-stimulated HUVECs

Stress fibers are contractile actomyosin bundles in endothelial cells that play a role in contraction and increase in intracellular gaps (Bogatcheva & Verin, 2008). Under normal conditions, F-actin is distributed throughout cells with localization on the cellular periphery (Figure 4A). Upon LPS stimulation, cytoskeleton rearrangement occurs with formation of stress fibers (4B), which are characterized as F-actins scattered over the cells as shown. HUVECs pretreated with tHGA (Figure 4C) and dexamethasone (Figure 4D) were shown to have less amount of stress fibers and maintained strong localization of peripheral F-actin in comparison to untreated cells.

Figure 4: tHGA reduces stress fibers in LPS-stimulated HUVECs. Treated HUVECs were stained with Alexa-Fluor 488 Phalloidin and Hoescht 33258 prior to viewing under 400x magnification. F-actin is normally localized at the peripherals of the cells, forming dense peripheral bands (indicated with yellow arrow) (A). The bands were disrupted and rearranged into stress fibers upon stimulation with LPS (B). Cells with high amount of stress fibers are marked with white arrow. Pretreatment with 20 µM tHGA (C) or 10 µM Dexamethasone elicited strong preventive effect on F-actin rearrangement, where dense peripheral bands were still abundantly found in the cells.



3.5 tHGA

Cell adhesion molecules (CAMs) play major role in mediating the adhesion of monocytes towards HUVECs. The expression of the proteins are upregulated with stimulation of LPS. Pretreatment of tHGA at 20μM and 5μM was found to be effective in suppressing the expression of the proteins (Figure 5). Such inhibitory effect of tHGA may be one of the many factors that causes decreased monocyte adhesion and migration, ultimately exhibiting barrier protective effect on LPS-stimulated HUVECs.

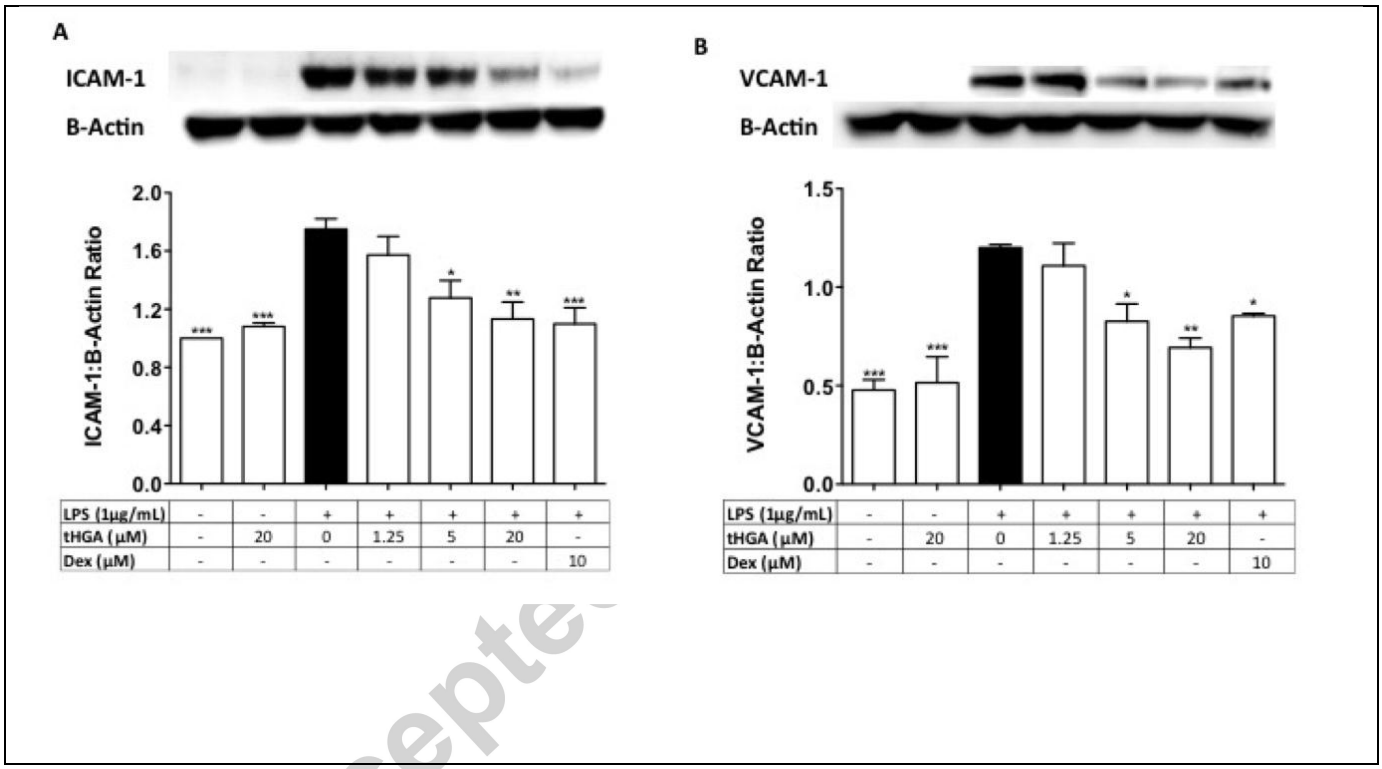


Figure 5: tHGA suppresses protein expression of ICAM-1 and VCAM-1. Pretreatment of 20 μM and 5 μM tHGA significantly suppressed the expression of ICAM-1 (A) and VCAM-1 (B). Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ significantly different from LPS-stimulated control without any treatment.

3.6 tHGA inhibits PGE_2, but does not inhibit MCP-1 release in LPS-stimulated HUVECs

Prostaglandin E_2 **(**PGE_2) and monocyte chemoattractant protein-1 (MCP-1) play important roles in LPS-stimulated inflammation. Upon stimulation by LPS, PGE_2 released by endothelial cells will upregulate inflammation (Engström et al., 2012) and enhance vascular dysfunction (Omori et al., 2014). Meanwhile, MCP-1 is an important chemoattractant protein which recruits circulating monocytes to the inflammatory region.

Figure 6 summarizes the effect of tHGA on PGE_2 and MCP-1 production by LPS-stimulated HUVECs. Both PGE_2 and MCP-1 concentration were significantly increased by 1.6 fold and 13.5 fold respectively upon stimulation with LPS. tHGA pretreatment effectively inhibited PGE_2 release. In particular, 20μM tHGA inhibited PGE_2 secretion to near non-stimulated levels. However, tHGA is ineffective in inhibiting MCP-1 secretion at all tested concentrations.

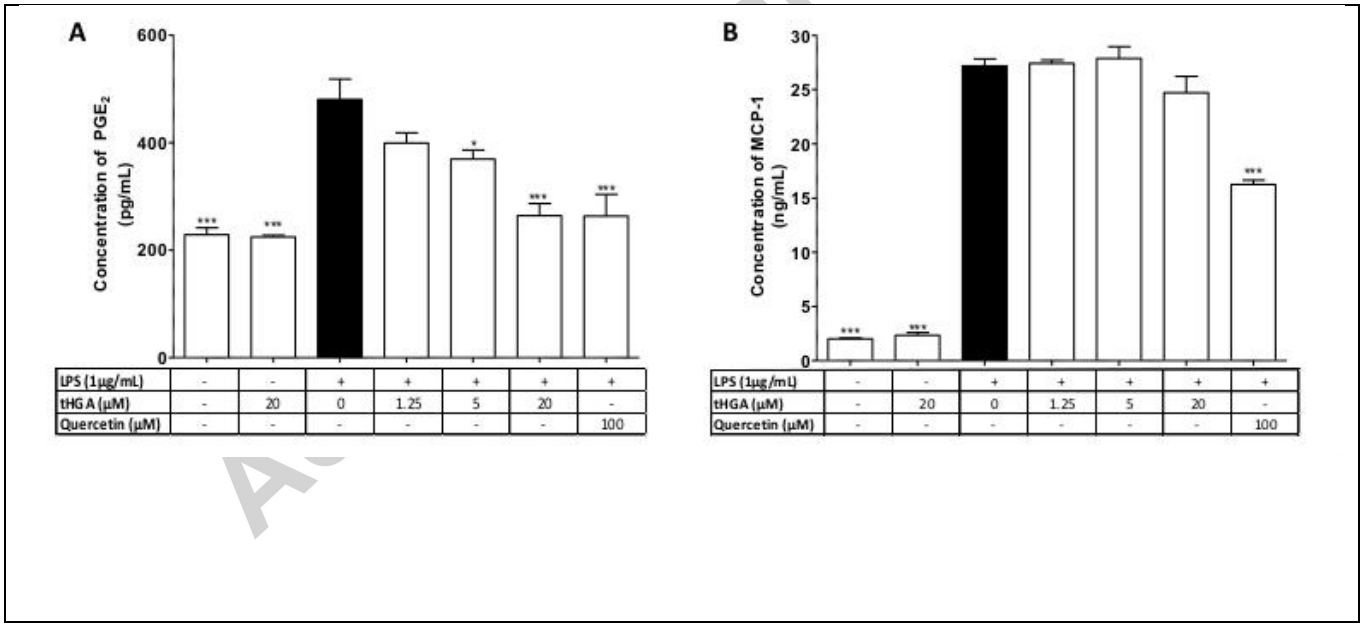


Figure 6: tHGA inhibits PGE_2 secretion but not MCP-1. tHGA significantly inhibited the secretion of PGE_2 secretion (A) at 5 and 20 μM, but not MCP-1 secretion (B). Results were expressed with mean ± S.E.M. of three independent experiments performed in triplicate. *** $P \leq 0.001$ significantly different from LPS-stimulated control without any treatment.

4. Discussion

The endothelium respond to physical and chemical signals through regulation of vascular tone, cellular adhesion, thrombo-resistance, smooth muscle cell proliferation, and vessel wall inflammation (Deanfield et al., 2007). When activation becomes exaggerated and sustained, the endothelial responses are detrimental to host and may lead to loss of selective permeability, resulting in vascular leakage that may eventually lead to shock (Lee and Slutsky, 2010). LPS from gram-negative bacteria can initiate the septic cascade through altered regulation of proadhesive properties and increased permeability (Aird, 2003). Its effect on the endothelium frequently leads to shock due to excessive fluid leakage. Therefore prevention of vascular leakage can be used as a method in managing LPS-stimulated systemic inflammation such as endotoxemia and sepsis (Goldenberg et al., 2011).

Phloroglucinol is a class of compound that is commonly found in plant extracts. Prior study reported that phloroglucinol alone was able to inhibit LPS-induced barrier disruption in human endothelial cells (Bae, 2012). Moreover, phlorotannin, which is a compound that contains phloroglucinols as common monomer unit, was found to possess barrier protective effect on HMGB1-mediated proinflammatory responses (Kim et al., 2012). The compound of interest in this study, 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), is one of the marker compounds that is originally found in the young leaves of *Melicope ptelefolia*. It has been reported to exhibit several anti-inflammatory effects, including anti-asthmatic effect (Ismail et al., 2012) and dual LOX/COX inhibitory effect (Shaari et al., 2011). Inhibition on COX-2 has been found to be effective in improving the outcome of LPS-stimulated endothelial dysfunction. Therefore, the barrier protective effect of tHGA on LPS-stimulated endothelial barrier dysfunction was explored in this study.

As upregulation of COX-2 derived PGE_2 secretion has been observed to promote alteration of barrier integrity in LPS-stimulated endothelial cells (Engström et al., 2012), we examined the effect of tHGA on PGE_2 secretion released by LPS-stimulated HUVEC monolayers. The results demonstrated that PGE_2 secretion was strongly inhibited by tHGA. One possible explanation for this inhibition could be the dual inhibitory effect of tHGA on 5-LOX and COX expression (Shaari et al., 2011). LPS stimulates cells via the TLR4 receptor, which subsequently initiates the arachidonic acid cascade. In order to synthesize PGE_2, COX-2 plays a major role in converting arachidonic acid to prostaglandin H_2 (PGH_2), which is the precursor of PGE_2. Therefore, it is hypothesized that tHGA may have inhibited COX-2 activity, which subsequently reduced production of PGH_2 and lastly, PGE_2.

In LPS-stimulated endothelial barrier dysfunction, inflamed endothelia are known to express high levels of MCP-1 and cell adhesion molecules (CAMs) to facilitate recruitment and binding of monocytes to the endothelium to initiate the extravasation process (Sprague & Khalil, 2009). As adhesion events often happen early in the inflammatory process and are essential in the regulation of extravasation, thus blocking the adhesion events may disrupt the leukocyte extravasation and all downstream events to prevent leukocytes from entering into the affected tissue (Simmons, 2006). In this study, tHGA was shown to reduce adhesion of activated U937 on LPS-stimulated HUVECs, as well as the subsequent transendothelial migration of U937 through LPS-stimulated HUVECs. Such results suggest that tHGA may have effects on molecules that play important roles in mediating monocyte adhesion and transendothelial migration, namely MCP-1 and CAMs.

Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (Deshmane et al., 2009). It is interesting to know the above barrier protective effects exerted by tHGA is solely due to inhibition on the adhesion events that further disrupts subsequent leukocyte transendothelial migration, or also partly due to inhibition on

the secretion of MCP-1 by activated endothelium. Therefore, we investigated the effect of tHGA on MCP-1 secretion through ELISA. Interestingly, our findings demonstrated no effects of tHGA upon MCP-1 secretion by LPS-stimulated HUVECs. As leukocyte extravasation can be resulted from morphological changes of the EC layer itself or/and inhibition of chemoattractants that target the transmigration of leukocytes (Schenkel et al., 2002), the mode of action of tHGA in endothelial dysfunstion may not MCP-1-dependent. Instead, tHGA might protect the EC layer itself through attenuation of LPS-stimulated CAMs expression and endothelial hyperpermeability.

We therefore examined the effects of tHGA on another critical factor that affects adhesiveness of endothelial cells: CAMs. Two types of CAMs have been found to play major role in mediating firm adhesion of circulating leukocytes towards the endothelial monolayer: ICAM-1 and VCAM-1. Both the CAMs are known to bind specifically to the integrins that are expressed in circulating monocytes, which would lead to firm adhesion of monocytes to endothelium (Muller, 2009). Under normal condition, both CAMs are expressed at low level by endothelial cells, and external stimulation such as TNF-α and LPS significantly increase the expression of these proteins. Therefore, we examined the effects of tHGA on the protein expression of ICAM-1 and VCAM-1 in LPS-stimulated HUVECs in this study. Our results from Western Blot analysis showed that tHGA strongly decreased the protein expression of both LPS-stimulated ICAM-1 and VCAM-1. As explained above, lowered expression of CAMs leads to reduction of the endothelium-monocyte interaction and prevention of extravasation. This therefore leads to our conclusion that the endothelial barrier protective effect of tHGA is primarily due to suppression of CAMs expression, but not through MCP-1-dependent chemoattraction.

During endotoxemia, vascular permeability is significantly increased in order to facilitate movement of protein and cells through the blood vessels. It is one of the hallmarks of endothelial barrier dysfunction. Our results have demonstrated that tHGA at 5 and 20 μM are effective in inhibiting both

EBA permeation and Na-F permeation. In particular, vascular dysfunction for HUVEC treated with 20 μM tHGA is near to basal level. These results suggested that tHGA is potentially effective in lowering endothelial vascular permeability via both transcellular and paracellular routes.

This finding was further confirmed by another experiment which demonstrated decreased stress fiber formation in LPS-stimulated HUVECs following pretreatment with tHGA. Vascular integrity is directly related to the cytoskeletal elements and cell-cell contact protein complexes. LPS activates several cytoskeletal rearranging pathways, such as the GEF-H1/Rho pathway which may lead to cytoskeletal rearrangement and pave ways to increase intercellular gaps and promote vascular permeability (Guo et al., 2012; Wolfson et al., 2011). This is most easily observed in stress fiber formation, which are actomyosin complexes that assist in the contraction of cells upon disruption of tight junctions and adherens junctions. The downregulation of stress fibers may be partly responsible for the barrier protective effect of tHGA. Collectively, inhibition on CAMs expression, endothelial permeability and F-actin cytoskeleton rearrangement indicates that tHGA acts directly on the HUVECS to elicit barrier protective function.

5. Conclusion

The inhibition of vascular endothelial inflammatory responses is considered a promising target for the treatment of many vascular diseases such as atherosclerosis, shock, heart attack and sepsis (Lee et al., 2012). Herein we described the effect of tHGA on LPS-stimulated HUVECs, in which tHGA is able to reduce the expression of adhesion proteins, formation of stress fibers, permeability, adhesion and leukocyte transmigration. Therefore as a whole, it is safe to conclude that tHGA is a potent anti-inflammatory compound with barrier protective potential. With such, the compound may have potential in treating diseases with LPS as the etiology. The mechanisms behind the endothelial barrier protective

properties of tHGA including its effect on mediators and the specific molecular target of tHGA that leads to preservation of the barrier integrity should also be studied. In additionally, the compound should be studied for its effect on *in vivo* disease models to maximize the compound's potential as a potential therapy for treating inflammatory diseases.

Conflict of Interests

The authors do not have any conflict of interests regarding the publication of this paper.

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