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Research Article
Suppressive Effects of Zingerone on Polyphosphate-Mediated Vascular Inflammatory Responses

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Abstract

Background and Objective: Human endothelial cells-derived polyphosphate (PolyP) is one of the pro-inflammatory mediators as suggested by the previous reports. Zingerone (ZGR), a phenolic alkanone isolated from ginger, has been reported to possess various pharmacological activities. This study was undertaken to investigate whether ZGR can modulate PolyP-mediated inflammatory responses in human umbilical vein endothelial cells (HUVECs) and in mice. **Materials and Methods:** After HUVECs or mice were activated with PolyP, cells or mice were post-treated with ZGR. The anti-inflammatory activities of ZGR were determined by measuring permeability, leukocytes adhesion and migration and activation of pro-inflammatory proteins in PolyP-activated HUVECs and mice. In addition, the beneficial effects of ZGR on survival rate in PolyP-injected mice. Statistical relevance was determined by one-way analysis of variance (ANOVA). P values less than 0.05 were considered to indicate significance. **Results:** It was found that ZGR inhibits PolyP-mediated barrier disruption, the expressions of cell adhesion molecules and leukocyte to HUVEC adhesion/migration. Interestingly, PolyP-induced NF-κB activation and the productions of TNF-α and IL-6 were inhibited by ZGR in HUVECs. These anti-inflammatory functions of ZGR were confirmed in PolyP injected mice. **Conclusion:** In conclusion, based on the anti-inflammatory effects of ZGR in PolyP-mediated septic response, ZGR have therapeutic potential for various systemic inflammatory diseases.

Key words: Barrier integrity, vascular permeability, phenolic alkanone, polyphosphate, zingerone

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Inorganic polyphosphate (PolyP) is a polymer formed by phosphate (Pi) residues linked by high-energy phosphoanhydride bonds[1]. Existing since prebiotic times, PolyP has been present in all cells from early in evolution and PolyP has been detected in human gingival fibroblasts, osteoblasts, erythrocytes and peripheral blood mononuclear cells[2-5]. Although the biological functions of polyP have been mainly investigated in microorganisms, previous studies suggest that polyP participates in numerous biological processes in mammalian systems, such as, in apoptosis, blood coagulation, cell proliferation and inflammation[2-5]. Previous reports showed the pro-inflammatory activities of polyP in human vascular endothelial cells and in mice[5-6]. These activities included the upregulation of vascular permeability, leukocytes to vascular endothelial cell adhesion/migration and modulations of the expressions of cell adhesion molecules, such as, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin[5]. Recently, it was reported the pro-inflammatory functions of PolyP in mice and human endothelial cells such as the hyperpermeability, behaviors (adhesion and migration) of leukocytes toward endothelial cells, upregulation of the expressions of cell adhesion molecules[5-8].

The herbal plant *Zingiber officinale* is a natural dietary spice with potent anti-inflammatory, antioxidative and anticancer properties[9]. Zingerone (ZGR) [4-(4-Hydroxy-3-methoxyphenyl) butan-2-one] is a stable active component of dry ginger rhizome[10] that has been reported to exhibit various pharmacological activities such as anti-inflammatory and anti-apoptotic effects and to confer protection from myocardial infarction and irritable bowel disorder[11-14]. However, the biological functions of ZGR on PolyP-induced severe vascular inflammatory activities have not been studied yet. The purpose of this study is to determine the cyto-protective effects of ZGR on PolyP-mediated severe inflammatory responses.

MATERIALS AND METHODS

This study was carried out, during 2016-2017, in the Biochemsitry and Cell Biology labs of College of Pharmacy in Kyungpook National University, Daegu, Republic of Korea. All chemicals and reagents used were of analytical grade obtained from various other commercial sources.

Reagents: PolyP65, ZGR, Evans blue, crystal violet, 2-Mercaptoethanol, polyethylene-glycolated (PEG)-catalase and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA). Stock solution (1 mM or 2 mM) of ZGR (anthocyanidin) or P3G (anthocyanin) was stored in -80°C until use and used stock solution was not returned to the freezer.

Animals and husbandry: Male mice (strain, C57BL/6; old, 6-7 weeks; average weight, 27 g) were obtained from Orient Bio Co. (Sungnam, Republic of Korea) and were used after their 12 days of acclimatization. The mice were maintained as described previously by Bae *et al.*[15] and Ku *et al.*[16] and treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University (IRB No; KNU 2016-54).

Cell culture: Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained using a previously described method[16-18]. HUVECs were used at cell culture passages 3-5. Human neutrophils were freshly isolated from whole blood (15 mL) obtained by venipuncture from five healthy volunteers and maintained as previously described by Jung *et al.*[19] and all experimental protocols (KNUH 2012-01-010) were approved by the Institutional Review Board of Kyungpook National University Hospitals (Daegu, Republic of Korea).

Permeability assay *in vitro*: In order to examine any changes in vascular permeability in response to increasing concentrations of ZGR, the flux of Evans blue-bound albumin was measured, as described previously by Bae and Rezaie[20]. Briefly, confluent HUVEC monolayers were treated with increasing concentrations of ZGR for 6 h and then activated with PolyP (50 M) for 4 h.

Enzyme-linked immunosorbent assay (ELISA) of phosphorylated (phospho) p-38: The confluent monolayers of the HUVECs were treated with PolyP (50 M) for 4 h and then with without ZGR. A commercially available ELISA kit (Cell Signaling Technology, Inc., Danvers, MA, USA) was used to measure the expression levels of phospho p-38.

Immunofluorescence staining: Confluent HUVECs on glass coverslips that were coated with 0.05% Poly-L-Lysine were maintained for 2 days. The HUVECs were then activated with PolyP (50 M, 4 h) with or without ZGR (25 or 50 M, 6 h). The cytoskeletal staining was assessed as previously described by Lee *et al.*[21].

Levels of expression of the protein and mRNA of cell adhesion molecules (CAMs): The confluent monolayers of the HUVECs were treated with PolyP (50 M) for 16 h for VCAM-1 and ICAM-1 or 24 h for e-selectin and then with without ZGR. A whole-cell ELISA was performed to determine the levels of expression of the ICAM-1, e-selectin and VCAM-1 proteins on the HUVECs, as previously described by Che *et al.*[22] and Bae and Bae[23]. For the real-time polymerase chain reaction, RNA was isolated with TRI Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The real-time polymerase chain reaction was performed as previously described by Lee and Bae[8].

Cell-cell adhesion assay: The cellular adhesion of purified human neutrophils to the HUVECs was tested by fluorescent labeling, as described previously by Bae and Bae[23]. Briefly, after the neutrophils were labeled with fluorescein, confluent HUVECs were activated with PolyP (50 M) for 4 h and then incubated with ZGR for another 6 h. The percentage of adherent neutrophils was calculated as previously described by Bae and Bae[23].

***In vitro* migration assay:** The migration of purified human neutrophils to the HUVECs was evaluated as previously described by Kim *et al.*[24]. After HUVECs were activated with PolyP (50 M) for 4 h followed by treating ZGR for 6 h, purified human neutrophils were then applied to the upper chamber and the migration index was measured as previously described by Kim *et al.*[24]. The results are presented as Migration Indices.

***In vivo* permeability and leukocyte migration assay:** The mice were treated with PolyP (6.5 µg/mouse, intravenous administration) or 0.5% dimethyl sulfoxide (DMSO), which was used as a control. The mice were then intravenously administered ZGR and 1% Evans blue dye solution in normal saline was injected after 4 h. The vascular permeability and leukocyte migration were determined as previously described by Bae *et al.*[5] and Lee *et al.*[25].

ELISA for nuclear factor (NF)-κB, extracellular signal-regulated kinase (ERK)1/2, interleukin (IL)-6 and tumor necrosis factor (TNF)-α: Commercially available ELISA kits were used to determine the levels of expression of total and phospho-NF-κB p65 (# 7174 and # 7173, Cell Signaling Technology, Inc.) and total/phospho ERK1/2 (R and D Systems, Inc., Minneapolis, MN, USA) in the nuclear lysates of HUVECs and the levels of IL-6 and TNF-α (R and D Systems, Inc.) in the cell culture supernatants of the HUVECs.

PolyP-induced lethal model: PolyP (6.5 g/mouse) in DMSO or 0.5% DMSO, which was a control, were intravenously injected into the mice. At 12 h or 50 h after the PolyP65 injection, male C57BL/6 mice were administered ZGR. Animal survival was monitored every 6 h after the PolyP65 injections for 132 h. All the animals were treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University.

Measurements of organ injury markers: Plasma levels of aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were measured using commercial assay kits (Pointe Scientific, Linclon Park, MI).

Statistical analysis: All experiments were independently performed a minimum of three times. Values were expressed as the Mean±Standard Deviation (SD). The statistical significance of differences between test groups was evaluated by SPSS for Windows, version 16.0 (SPSS, Chicago, IL). Statistical relevance was determined by one-way analysis of variance (ANOVA) and Tukey's post-test. Values of $p<0.05$ were considered to indicate statistical significance. The survival of CLP-induced sepsis outcomes was assessed using Kaplan-Meier analysis[15,26].

RESULTS AND DISCUSSION

Effect of ZGR on PolyP-mediated vascular barrier disruption: Vascular permeability was assessed to test the effects of ZGR on the PolyP-induced disruptions of the vascular barrier as the endothelial barrier integrity is cleaved by PolyP[5-6]. Our previous studies reported the PolyP parameters (50 M and 4 h) that optimize the disruption of endothelial integrity[5-6], which is consistent with the current study (Fig. 1a and b). The cells were activated with PolyP (50 M) for 4 h and then various concentrations of ZGR for 6 h. The results showed the inhibitory effects of ZGR on the PolyP-mediated hyperpermeability, with the optimal dose occurring at concentrations above 10 M (Fig. 1c) and there was no additional barrier protective effect over 50 M ZGR (data not shown). Furthermore, ZGR alone (50 M) did not alter the barrier integrity of the HUVECs (Fig. 1c). Next, to confirm in vitro data, ZGR was intravenously injected into mice with PolyP-mediated hyperpermeability. The results showed that PolyP enhanced vascular permeability and this was suppressed by ZGR (Fig. 1d). Because the average blood volume is 72 mL kg^{-1} [27] and the average weight of the mice

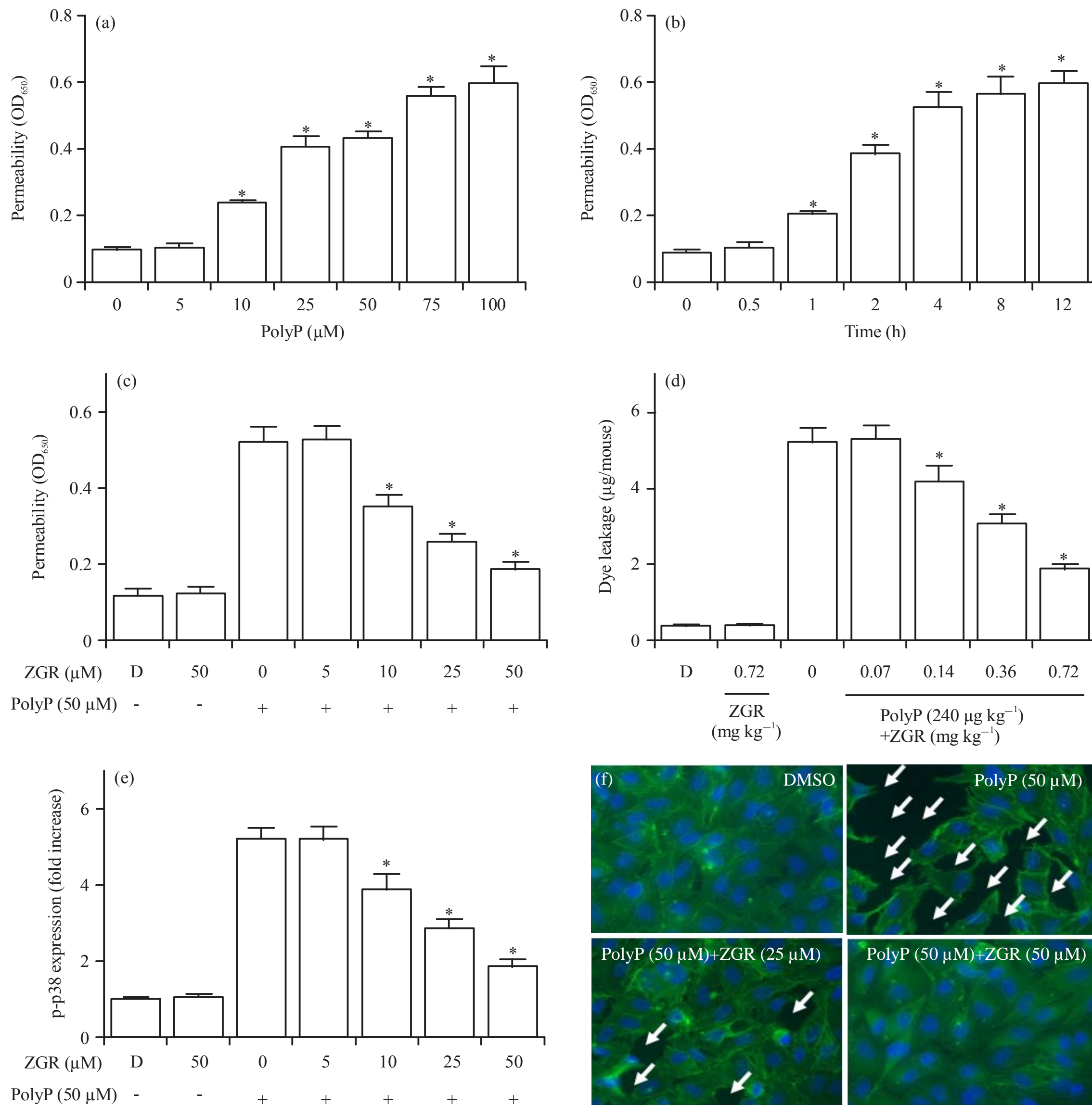

Fig. 1(a-f): Effects of ZGR on PolyP-induced barrier disruption *in vitro* and *in vivo*. (a, b) HUVECs were incubated with indicated concentrations of PolyP for 6 h (a) or with 50 M PolyP for indicated periods (b) followed by measuring permeability, (c) Effect of various concentrations of ZGR on PolyP-induced (50 M, 4 h) barrier disruption was monitored by the flux of Evans blue bound albumin across HUVECs, (d) Effect of ZGR on PolyP (3.5 g/mouse, i.v.)-induced vascular permeability in mice was examined by the flux of Evans blue in mice (expressed g/mouse, n = 5), (e) HUVECs were treated with different concentrations of ZGR for 6 h, after activation with PolyP (50 M, 4 h). The effects of ZGR on PolyP-mediated phosphor-p38 expression were determined by ELISA and (f) Staining for F-actin. HUVECs monolayers grown on glass coverslips were stimulated with PolyP (50 M, 4 h), followed by treatment with ZGR for 6 h and immunofluorescence staining for F-actin. Arrows indicate intercellular gaps. "D" in each X-axis means vehicle (0.5% DMSO) only. Results are expressed as the Mean±SD of three independent experiments. *p<0.05 versus 0 (a-b) or PolyP (c-e)

that were used in this study was 27 g, the amount of ZGR (0.07, 0.14, 0.36, or 0.72) injected was equivalent to 5, 10, 25, or 50 M in peripheral blood.

Vascular inflammatory inducers, such as lipopolysaccharide and high-mobility group box 1 protein, mediate inflammatory responses by activating p38 mitogen-

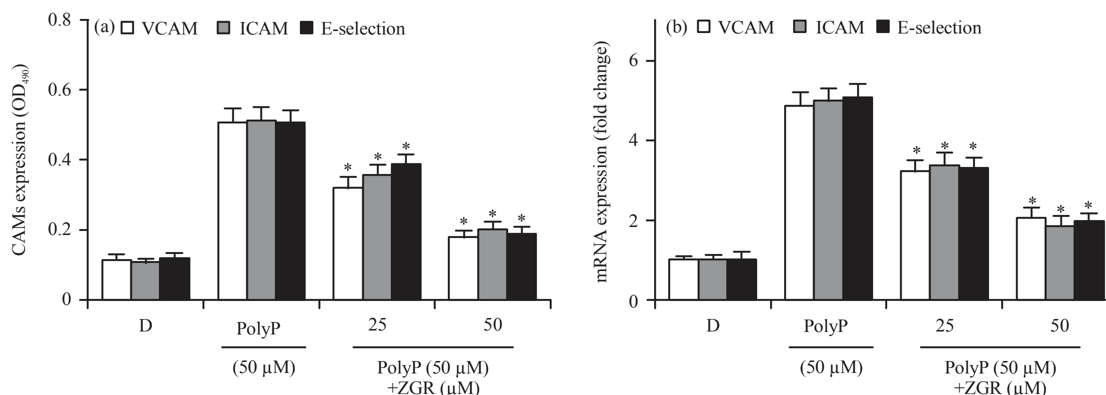

Fig. 2(a-b): Effects of ZGR on PolyP-induced CAMs expression in HUVECs, (a) PolyP-mediated (50 M) expression of VCAM-1 (white bar), ICAM-1 (gray bar) and E-selectin (black bar) in HUVECs was analyzed after treating monolayers with ZGR by whole cell ELISA and (b) The same as (a) except that transcription level (mRNA) was analyzed. Results are expressed as the Mean±SD of three independent experiments. *p<0.05 versus PolyP

activated protein kinase (MAPK)[28-29]. Therefore, the effects of ZGR on the PolyP-induced activation of p38 MAPK were determined. The results revealed that ZGR inhibited the PolyP-induced upregulation of phospho p38 expression (Fig. 1e). Previous studies have indicated the importance of cytoskeletal proteins for maintaining cell integrity and shape[30] and the involvement of vascular integrity in the detachment of cell-cell contact and redistribution of the actin cytoskeleton[31-32]. Thus, the effects of ZGR on PolyP-mediated actin cytoskeletal arrangement in HUVECs were examined by staining the HUVECs with fluorescein phalloidin-labeled F-actin. Compared to the control HUVECs that displayed an irregular distribution of F-actin, the disruption in the barrier integrity that was induced by treatment with PolyP (50 M) was demonstrated by the formation of paracellular gaps in the HUVECs and these were reduced by treatment with ZGR (Fig. 1f). To exclude the possibility that the barrier-protecting effects of ZGR were due to the cellular cytotoxicity of ZGR, cellular viability assays were conducted. Data showed that ZGR was not cytotoxic in the HUVECs at concentrations up to 100 μM.

Because the high morbidity and mortality that are seen in patients with serious inflammatory diseases result from the disruption of vascular integrity[33] and because the reagents used to treat a number of inflammatory diseases are designed to inhibit vascular hyperpermeability[34], our results indicated the potential of ZGR as a therapeutic agent in various vascular inflammatory diseases.

Effects of ZGR on PolyP-mediated CAMs expression, neutrophils adhesion and migration: It is well known that CAMs, such as VCAM-1, ICAM-1 and E-selectin, play a role in cell adhesion to the vascular endothelium, which precedes the extravasation of cells and the development of vascular inflammation[35-36]. Therefore, inhibiting the expression of CAMs in vascular endothelial cells is considered to be a promising therapeutic approach for treating vascular inflammatory diseases. Previous studies have demonstrated that PolyP mediates inflammatory responses through the increase of the cell-surface expression of CAMs, such as VCAM-1, ICAM-1 and E-selectin on the surfaces of endothelial cells, thereby promoting the adhesion and migration of leukocytes across endothelium to sites of inflammation[5-6]. In this study, it was determined the effects of ZGR on the levels of expression of CAMs and on the adhesion and migration of leukocytes toward HUVECs, which were both affected by PolyP. The results showed that ZGR suppressed the increases in the levels of protein and transcript expression of CAMs (Fig. 2a and b). In addition, the enhancement in the expression of CAMs correlated with the increased binding and migration of leukocytes to PolyP-treated HUVECs and this was inhibited by ZGR treatment in a concentration-dependent manner (Fig. 3a-c). In order to confirm these results *in vivo*, it was examined the effects of injected ZGR on PolyP-induced leukocyte migration in mice. PolyP increased the number of migrated leukocytes in the peritoneal cavities of mice and this was reduced by ZGR treatment (Fig. 3d). Collectively, the results of this study showed that ZGR downregulated PolyP-mediated vascular inflammatory responses by inhibiting the augmentation by PolyP of inflammatory signaling pathways, such as the adhesion and migration of leukocytes to inflamed endothelium.

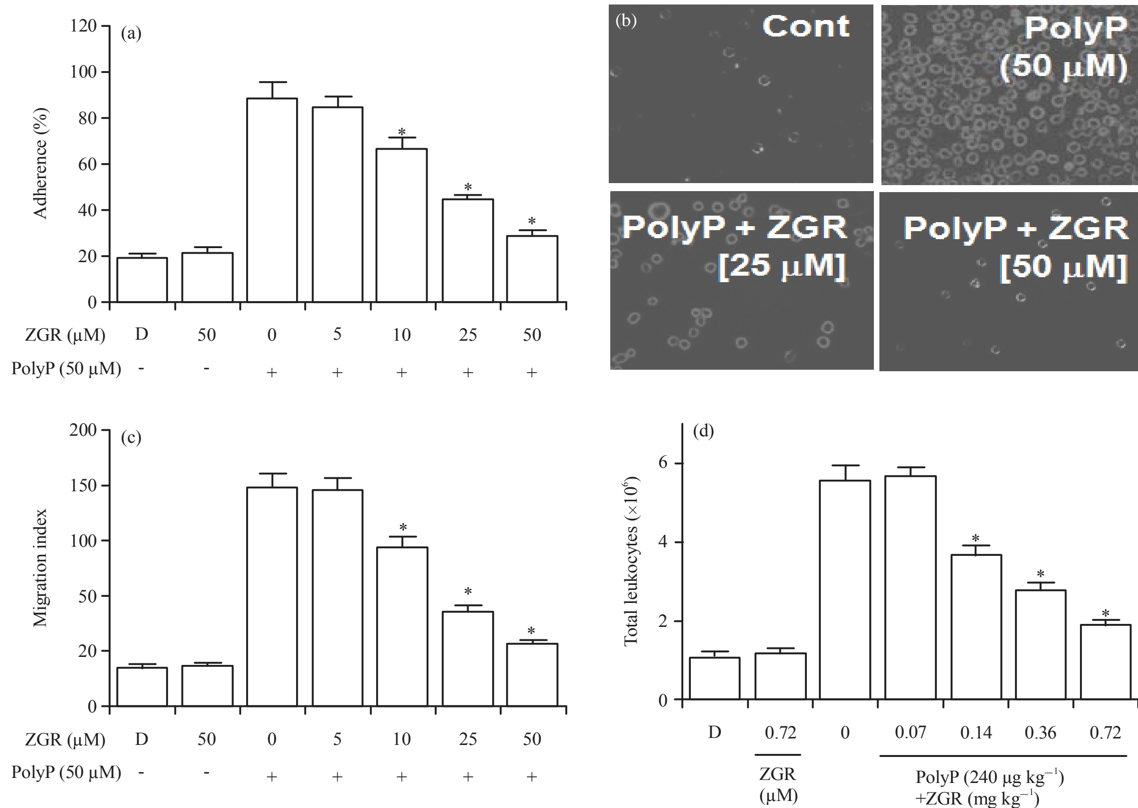

Fig. 3(a-d): Effects of ZGR on PolyP-induced cell adhesion and TEM, (a) PolyP-mediated (50 M) adherence of neutrophils to HUVEC was analyzed after treating cells with ZGR, (b) Representative photomicrographs of neutrophils adhesion to HUVECs, (c) PolyP-mediated (50 M) migration of neutrophils through HUVEC monolayers was analyzed after treating cells with ZGR and (d) Effect of ZGR on PolyP (3.5 g/mouse, i.v.)-induced leukocyte migration in mice (expressed $\times 10^6$, n = 5). Results are expressed as the Mean$\pm$SD of three independent experiments. *p<0.05 versus PolyP

Effects of ZGR on PolyP-stimulated activation of NF-κB/ERK and production of IL-6/TNF-α: NF-κB activation is required for pro-inflammatory responses and the three most important providers of inflammatory signals in endothelial cells are NF-κB, TNF-α and IL-6[37-39]. Therefore, it was hypothesized that ZGR might inhibit the expression or activity of these pro-inflammatory molecules. To investigate the potential effects of ZGR on the production of the pro-inflammatory cytokines, IL-6 and TNF-α, HUVECs were incubated with ZGR for 6 h after PolyP activation, followed by the measurement of IL-6 and TNF-α levels in the culture media via ELISA. Levels of TNF-α and IL-6 showed an increase in PolyP-stimulated endothelial cells; these increases were significantly reduced ZGR (Fig. 4a, b), indicating that ZGR can regulate the most important signals that induce pro-inflammatory responses in human endothelial cells. Activation of NF-κB and ERK1/2 is required for pro-inflammatory responses[38,40-41] and PolyP is known to activate NF-κB and ERK1/2 in vascular inflammatory responses[5-6]. Thus, it was hypothesized that the activation and expression of these proinflammatory molecules were suppressed by ZGR. To confirm this hypothesis, the levels of activation and expression of these proinflammatory molecules were measured with ELISA in PolyP-activated and ZGR-treated HUVECs. The results showed that the increased levels of protein expression of TNF-α and IL-6 (Fig. 4a and b) and the increased activation of NF-κB and ERK1/2 (Fig. 4c and d) that were induced by PolyP were reduced by ZGR. Therefore, these results suggested that ZGR can control important vascular inflammatory signaling pathways by regulating the molecules involved.

Protective effect of ZGR in the polyP-induced lethal model: Finally, it was hypothesized that ZGR would prevent PolyP-mediated lethality in mice. To confirm this, mice were administered with ZGR after PolyP injections. The results showed that treatment with a single dose of ZGR (0.72 mg kg^{-1}, 12 h after PolyP injection) did not prevent PolyP-induced death (data not shown). Thus, ZGR was

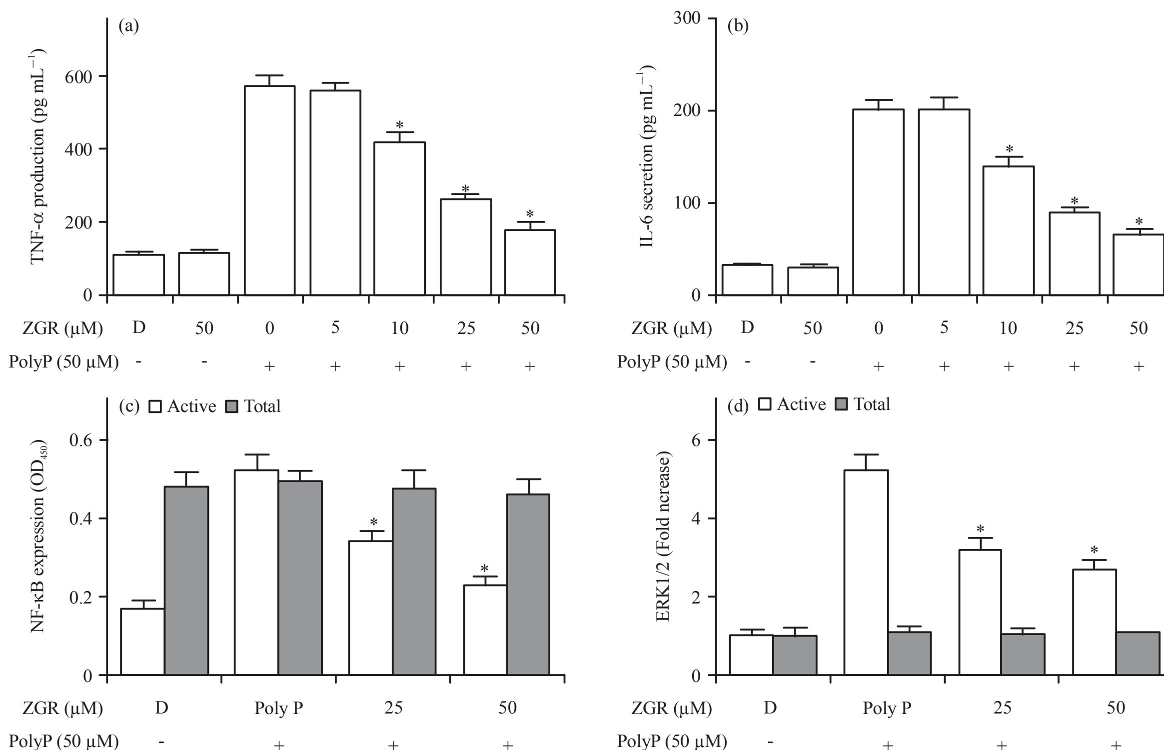

Fig. 4(a-d): Effects of ZGR on PolyP-stimulated activation of NF-κB/ERK and production of IL-6/TNF-α. PolyP-mediated (50 M)-mediated production of TNF-α (a) or IL-6 (b) in HUVECs was analyzed after treatment of cells with the indicated concentrations of ZGR for 6 h, (c) PolyP-mediated (50 M)-mediated activation of phospho-NF-κB p65 (white bar) or total NF-κB p65 (black bar) in HUVECs was analyzed after treatment of cells with ZGR for 6 h and (d) The same as (c) except that phospho-ERK1/2 (white box) or total ERK1/2 (black box) in HUVECs was analyzed. *p<0.05 vs. PolyP only

administered twice (once at 12 h and then at 50 h after the PolyP injection), which resulted in an increase in the survival rate from 0-50% in the Kaplan-Meier survival analysis (Fig. 5a, $p<0.0001$) and there was no additional survival effect over 0.72 mg kg^{-1} ZGR (data not shown). And, when ZGR was administrated orally, the survival effects of ZGR were worse than those of intravenous injection (Fig. 5b, $p<0.0001$). If ZGR was ingested as edible foods, an amount greater than the calculated amounts of these compounds is needed for the following reasons: 1) Large differences exist between intravenous injection and the oral route; 2) After ingesting foods, several pharmacological processes are required for the compound to reach the blood stream, such as absorption, distribution, metabolism and excretion (ADME) and 3) The entire content of ZGR in foods is not absorbed. In order for a consumed compound to be utilized by the body's vascular system, the following 4 criteria, which represent the disposition of a pharmaceutical compound within an organism, should be met the ADME. These 4 criteria influence the compound levels and the kinetics of exposure of the tissues to the compound; hence, these criteria influence the performance and pharmacological activity of the compound.

Because the liver and kidney are major target organs of systemic inflammatory diseases and multiple organ failure is caused by systemic inflammatory diseases, such as sepsis and septic shock[42], it was examined the plasma levels of tissue damage markers. As shown in Fig. 5c-f, ZGR reduced the polyP-induced increases in the plasma levels of alanine transaminase and aspartate transaminase (markers of hepatic injury, Fig. 5c) and creatinine and blood urea nitrogen (markers of renal injury, Fig. 5d and e). In addition, the levels of lactate dehydrogenase, which is a marker of tissue injury, were reduced by ZGR in PolyP-injected mice (Fig. 5f).

Sepsis is defined as Systematic Inflammatory Response Syndrome (SIRS) caused by infection[43]. Sepsis and severe sepsis (sepsis accompanied by acute organ dysfunction) are the leading causes of death in high-income countries and, despite advances in antibiotic therapies and intensive care, they remain the most common cause of death among critically ill patients in non-coronary Intensive Care Units (ICU)[44-45]. Xigris (Eli Lilly) and was approved in 2001 by the Food and

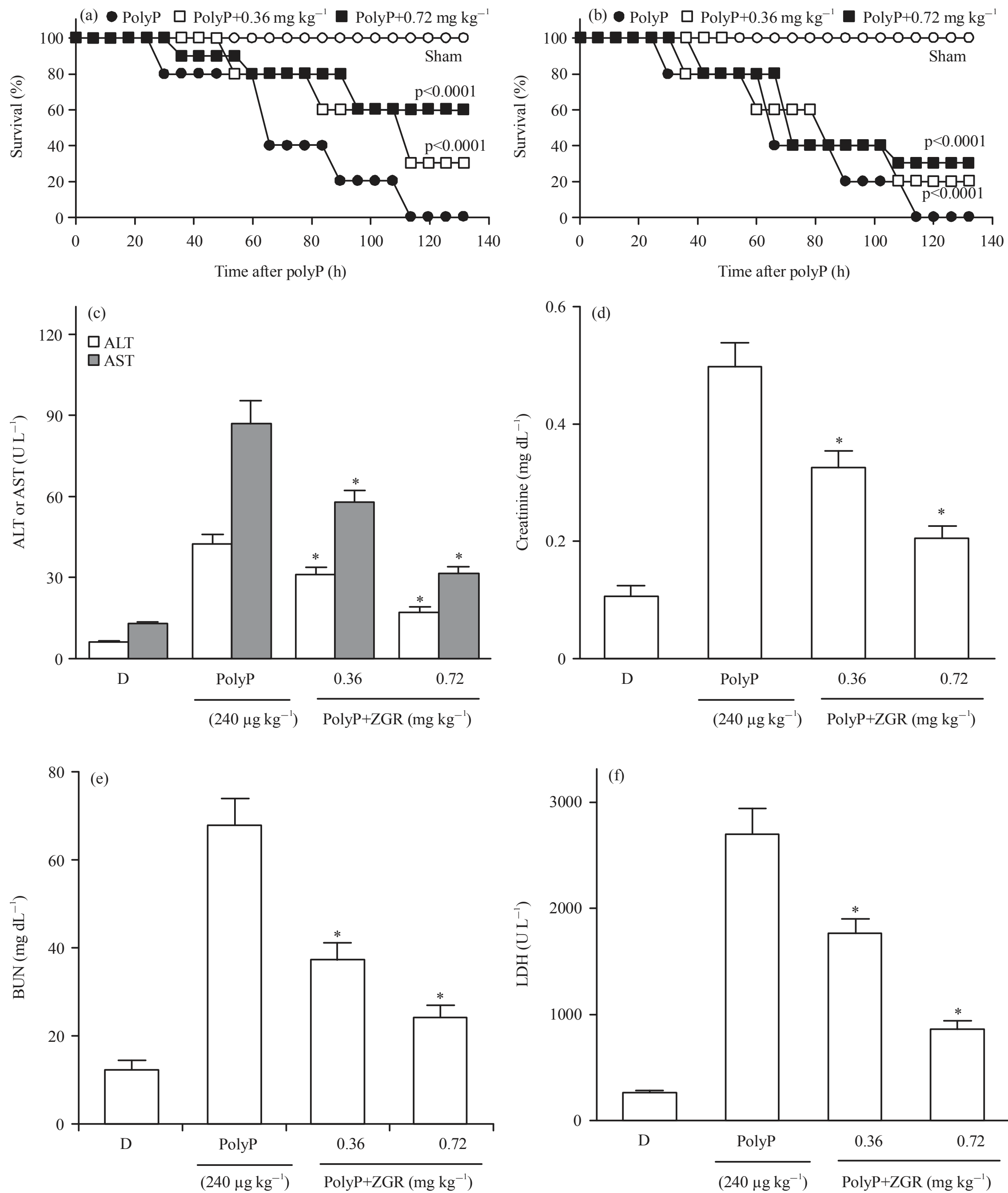

Fig. 5(a-f): Effects of ZGR on PolyP-induced lethality and organ damage markers, (a, b) Male C57BL/6 mice (n = 20) were administered ZGR at 0.36 mg kg^{-1} (□) or at 0.72 mg kg^{-1} (■) each by intravenously (a) or orally (b), 12 h and 50 h after PolyP (3.5 g/mouse, i.v.) injection. Animal survival was monitored every 6 h after PolyP injection for 132 h. PolyP-injected mice (●) and control mice (○) were administered 0.5% DMSO (n = 20). Kaplan-Meier survival analysis used for determination of overall survival rates versus PolyP treated mice. (c) The activity of hepatic injury markers AST and ALT, (d) Levels of renal injury markers creatinine, (e) BUN and (f) Tissue injury marker LDH levels were measured (n = 5) 72 h after PolyP injection. *$p<0.05$ vs. PolyP alone

Drug Administration (FDA) and in 2002 by the European Medicine Agency (EMA) for the treatment of severe sepsis and septic shock[46]. In October, 2011, Xigris was withdrawn from the market due to side effects and a lack of beneficial effects

on 28 day mortality in the PROWESS and septic shock (PROWESS-SHOCK) trials[47]. With the withdrawal of Xigris, there is a lack of drugs specifically approved for the treatment of severe sepsis.

The molecular mechanisms underlying the anti-inflammatory effects of ZGR on PolyP-mediated septic responses may be mediated by the suppression of PolyP-mediated hyperpermeability (Fig. 1c and d) through the inhibition of the activation of p38 (Fig. 1e). Furthermore, the inhibitory mechanisms of ZGR on the interaction of leukocytes with endothelial cells are mediated by the inhibition of the expression of CAMs, such as VCAM, ICAM and e-selectin (Fig. 2 and 3). The underlying mechanisms of these anti-inflammatory effects of ZGR involve the downregulation of the production of inflammatory cytokines (TNF-α and IL-6, Fig. 4a and b) and the activation of inflammatory transcriptional factors (NF-κB and ERK1/2, Fig. 4c and d).

CONCLUSION

Our results demonstrate that ZGR inhibited PolyP-mediated barrier disruption through increases in barrier integrity and inhibition of CAM expression. In addition, ZGR reduces human neutrophil adhesion and migration toward HUVECs. These barrier protective effects of ZGR were confirmed in a mouse model, in which treatment with ZGR resulted in a reduction in PolyP-induced mortality. The results of this study showed the protective activities of ZGR on the vascular barrier disruptions induced by PolyP in both human endothelial cells and mice. Therefore, these results suggested that ZGR is a potential candidate in the treatment of severe vascular inflammatory diseases.

SIGNIFICANCE STATEMENT

This study discovered the anti-septic effects of zingerone against polyphosphate-mediated septic responses that can be beneficial for development of sepsis drug candidate. This study will help the researcher to uncover the critical areas of vascular inflammatory diseases that many researchers were not able to explore. Thus, a new theory on anti-septic effects of natural compound and possibly other combinations, will lead to the development of new sepsis treatments.

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