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Research Article
Inhibitory Effects of Sulforaphane on Polyphosphate-mediated Septic Responses

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Abstract

Background and Objective: Sulforaphane (SFN), a natural isothiocyanate present in cruciferous vegetables, such as broccoli and cabbage, is effective in preventing carcinogenesis, diabetes and inflammatory responses. Human endothelial cell-derived polyphosphate (PolyP) is a proinflammatory mediator. This study was undertaken to investigate whether SFN can modulate PolyP-mediated inflammatory responses in Human Umbilical Vein Endothelial Cells (HUVECs) and mice. **Materials and Methods:** The anti-inflammatory activities of SFN were determined by measuring the permeability, leukocyte adhesion and migration and activation of proinflammatory proteins in PolyP-activated HUVECs and mice. In addition was determined the beneficial effects of SFN on survival rate in PolyP-injected mice. Statistical relevance was determined by one-way analysis of variance (ANOVA) and Tukey's post-test. **Results:** Data showed that SFN inhibits PolyP-mediated barrier disruption, cell adhesion molecule expression and leukocyte to HUVEC adhesion/migration. Notably, PolyP-induced NF-κB activation and TNF-α and IL-6 production were inhibited by SFN in HUVECs. These anti-inflammatory functions of SFN were confirmed in PolyP injected mice. **Conclusion:** Based on the anti-inflammatory effects of SFN in PolyP-mediated septic response, SFN could be considered a potential agent for the treatment of sepsis and other diseases in which PolyP is viewed as a therapeutic target.

Key words: Sulforaphane, polyphosphate, sepsis, barrier integrity, therapeutic target

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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 of phosphate (Pi) residues linked by high-energy phosphoanhydride bonds[1]. Existing since prebiotic times, PolyP is present in all cells from early evolution and it has been detected in human gingival fibroblasts, osteoblasts, erythrocytes and peripheral blood mononuclear cells[2-5]. Recently, it was reported that the proinflammatory functions of PolyP in mice and human endothelial cells, such as hyperpermeability, behavior (adhesion and migration) of leukocytes toward endothelial cells and upregulation of Cell Adhesion Molecule (CAM) expression[5-8].

Sulforaphane (SFN) is an organosulfur compound that exhibits anticancer and anti-diabetic properties in experimental models. It is found in cruciferous vegetables, such as broccoli, Brussels sprouts and cabbage[9]. Therefore, the increased consumption of cruciferous vegetables has been associated with a decreased risk of several degenerative and chronic diseases, including cardiovascular disease. The SFN has garnered particular interest as an indirect antioxidant owing to its extraordinary ability to induce the expression of multiple endogenous enzymes via the upregulation of nuclear factor E2-related factor-2 (Nrf2) function[9]. In addition, Heiss *et al.*[10] reported that SFN possesses anti-inflammatory properties, resulting in the downregulation of lipopolysaccharide (LPS)-stimulated inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and tumor necrosis factor (TNF)-α expression in RAW macrophages owing to the inhibition of DNA binding in NF-κB.

To the best of our knowledge, the biological functions of SFN on PolyP-induced severe vascular inflammatory activities have not been studied yet. In this study aimed to report the anti-inflammatory functions of SFN on PolyP-mediated severe inflammatory responses. It was showed that SFN inhibits PolyP-mediated barrier disruption, cell adhesion molecule expression and leukocyte to human umbilical vein endothelial cells (HUVECs) adhesion/migration. Notably, PolyP-induced NF-κB activation as well as TNF-α and IL-6 production was inhibited by SFN in HUVECs. These anti-inflammatory functions of SFN were confirmed in PolyP injected mice.

MATERIALS AND METHODS

This study was carried out, 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, SFN, Evans blue, crystal violet, 2-mercaptoethanol, polyethylene-glycolated (PEG)-catalase and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO, USA). Fetal Bovine Serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA). The stock solution (1 or 2 mM) of SFN (anthocyanidin) or P3G (anthocyanin) was stored at -80°C until use and the 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 used after 12 days of acclimatization. The mice were maintained as described previously[11-12] 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 HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained using a previously described method[12-14]. The HUVECs were used at cell culture passages 3-5. Human neutrophils were freshly isolated from whole blood (15 mL) by venipuncture from five healthy volunteers and maintained as previously described[15-17].

Permeability assay *in vitro*: To examine any changes in vascular permeability in response to increasing concentrations of SFN, the flux of Evans blue-bound albumin was measured as described previously[18]. Briefly, confluent HUVEC monolayers were treated with increasing concentrations of SFN 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 HUVECs were treated with PolyP (50 M) for 4 h and then with or without SFN. 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 SFN (20 or 30 M, 6 h). Cytoskeletal staining was assessed as previously described[19].

Expression levels of protein and mRNA of CAMs: The confluent monolayers of 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 or without SFN. A whole-cell ELISA was performed to determine the expression levels of ICAM-1, e-selectin and VCAM-1 proteins in HUVECs, as previously described[20-21]. For real-time Polymerase Chain Reaction (PCR), RNA was isolated with TRI Reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The real-time PCR was performed as previously described[8].

Cell-cell adhesion assay: The cellular adhesion of purified human neutrophils to HUVECs was tested by fluorescent labeling as described previously[21]. Briefly, after the neutrophils were labeled with fluorescein, confluent HUVECs were activated with PolyP (50 M) for 4 h and then incubated with SFN for another 6 h. The percentage of adherent neutrophils was calculated as previously described[21].

***In vitro* migration assay:** The migration of purified human neutrophils to HUVECs was evaluated as previously described[22]. After activation of HUVECs with PolyP (50 M) for 4 h, followed by treating SFN for 6 h, purified human neutrophils were applied to the upper chamber and the migration index was measured as previously described[22]. The results are presented as migration indices.

***In vivo* permeability and leukocyte migration assay:** The mice were treated with PolyP (6.5 g mouse^{-1}, intravenous administration) or 0.5% dimethyl sulfoxide (DMSO), which was used as a control. The mice were then intravenously administered SFN. Evans blue dye solution (1%) in normal saline was injected after 4 h. The vascular permeability and leukocyte migration were determined as previously described[5,23].

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 expression levels of total/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, as well as the levels of IL-6 and TNF-α (R and D Systems, Inc.) in the cell culture supernatants of HUVECs.

PolyP-induced lethal model: PolyP (6.5 g mouse^{-1}) in DMSO or 0.5% DMSO, which was a control was intravenously injected into the mice. At 12 or 50 h after the PolyP65 injection, male C57BL/6 mice were administered SFN. 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: The 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. The values were expressed as the Mean±Standard Deviation (SD). The statistical significance of differences between the 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 statistically significant. The survival of CLP-induced sepsis outcomes was assessed using Kaplan-Meier analysis[11,24].

RESULTS AND DISCUSSION

Effect of SFN on PolyP-mediated vascular barrier disruption: Noting that the vascular barrier integrity is disrupted by inflammatory mediators, a permeability assay was used to determine the effects of SFN on the maintenance of barrier integrity. To do this, HUVECs were activated with PolyP because previous studies reported the PolyP parameters (50 M and 4 h) that optimize the disruption of endothelial integrity[5-6]. The cells were activated by PolyP (50 M) treatment for 4 h and then treated with various concentrations of SFN for 6 h. The results showed the inhibitory effects of SFN on PolyP-mediated hyperpermeability, with the optimal dose occurring at concentrations above 10 M (Fig. 1a). There was no additional barrier protective effect above SFN concentration of 30 M. Furthermore, SFN alone (30 M) did not alter the barrier integrity of HUVECs (Fig. 1a). Next, to confirm *in vivo* data, SFN was intravenously injected into mice with PolyP-mediated hyperpermeability. The results showed that PolyP enhanced vascular permeability, which was suppressed by SFN (Fig. 1b). Because the average blood volume is 72 mL kg^{-1} [25] and the average weight of the mice used in this study was 27 g, the amount of SFN (0.07, 0.13, 0.26 or 0.39) injected was equivalent to 5, 10, 20 or 30 M in peripheral blood.

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

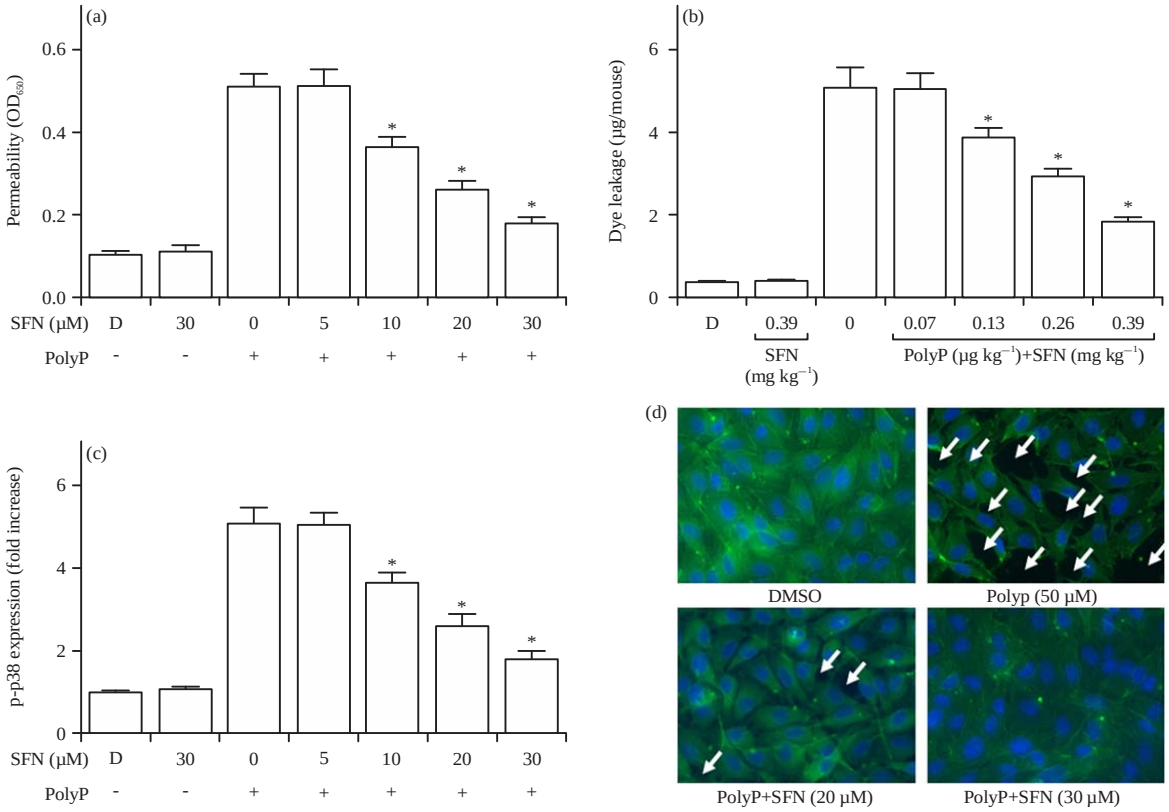

Fig. 1(a-d): Effects of SFN on PolyP-induced barrier disruption *in vitro* and *in vivo*, (a) Effect of various concentrations of SFN on PolyP-induced (50 M, 4 h) barrier disruption was monitored by the flux of Evans blue-bound albumin across HUVECs, (b) Effect of SFN on PolyP (3.5 g mouse^{-1}, i.v.) induced vascular permeability in mice was examined by the flux of Evans blue in mice (expressed g mouse^{-1}, n = 5), (c) HUVECs were treated with different concentrations of SFN for 6 h after activation with PolyP (50 M, 4 h). The effects of SFN on PolyP-mediated phospho-p38 expression were determined by ELISA and (d) Staining for F-actin. HUVEC monolayers grown on glass coverslips were stimulated with PolyP (50 M, 4 h), followed by treatment with SFN for 6 h and immunofluorescence staining for F-actin

Arrows indicate intercellular gaps, "D" in each X-axis means vehicle (0.5% DMSO) only, The results are expressed as the Means±SD of three independent experiments, *p<0.05 vs. PolyP

(MAPK)[26-27]. Therefore, the effects of SFN on PolyP-induced activation of p38 MAPK were determined. The results revealed that SFN inhibited the PolyP-induced upregulation of phospho p38 expression (Fig. 1c). Previous studies have indicated the importance of cytoskeletal proteins for maintaining cell integrity and shape[28] and involvement of vascular integrity in the detachment of cell-cell contact and redistribution of the actin cytoskeleton[29-30]. Thus, the effects of SFN on PolyP-mediated actin cytoskeletal arrangement in HUVECs were examined by staining HUVECs with fluorescein phalloidin-labeled F-actin. Compared to the control-treated HUVECs that displayed an irregular distribution of F-actin, HUVECs treated with PolyP (50 M) showed barrier integrity disruption, which was demonstrated by the formation of paracellular gaps and these gaps were reduced by SFN treatment (Fig. 1d). To exclude the possibility that the barrier-protecting effects of SFN were due to the cellular cytotoxicity of SFN, cellular viability assays were conducted. Data showed that SFN was not cytotoxic against HUVECs at concentrations up to 50 μM.

Because the high morbidity and mortality seen in patients with serious inflammatory diseases result from the disruption of vascular integrity[31] and the reagents used to treat a number of inflammatory diseases are designed to inhibit vascular hyperpermeability[32], these results indicated the potential of SFN as a therapeutic agent in various vascular inflammatory diseases.

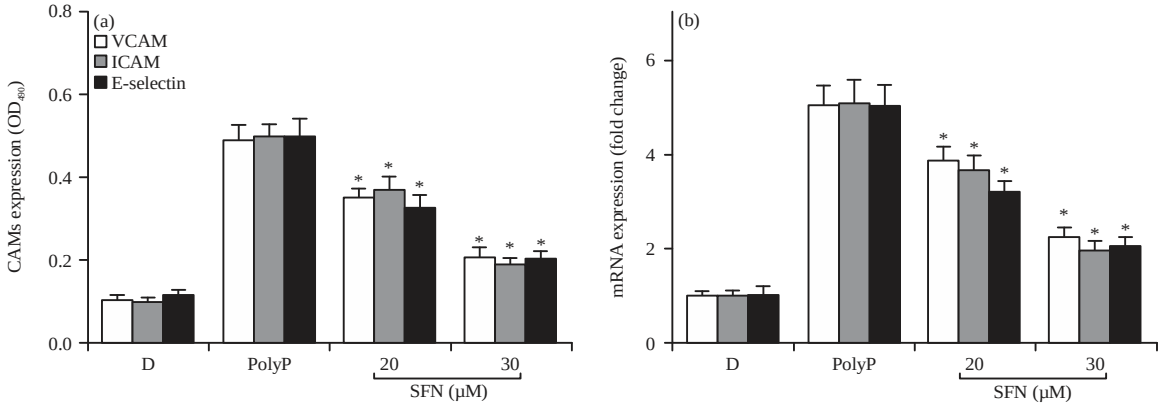

Fig. 2(a-b): Effects of SFN on PolyP-induced CAM expression in HUVECs, (a) PolyP-mediated (50 μM) expression of VCAM-1 (white bar), ICAM-1 (gray bar) and E-selectin (black bar) in HUVECs was analyzed after treating the monolayers with SFN using whole-cell ELISA and (b) The same as (a) except that transcription level (mRNA) was analyzed

The results are expressed as the Means±SD of three independent experiments, *p<0.05 vs. PolyP alone

Effects of SFN on PolyP-mediated CAM expression, neutrophil adhesion and migration: It is well known that CAMs, such as VCAM-1, ICAM-1 and selectin, play a role in cell adhesion to the vascular endothelium, which precedes the extravasation of cells and development of vascular inflammation[33-34]. 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 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 the endothelium to the sites of inflammation[5-6]. In this study, it was determined the effects of SFN on the expression levels of CAMs and the adhesion and migration of leukocytes toward HUVECs, which were both affected by PolyP. The results showed that SFN suppressed the increases in the levels of protein and transcript expression of CAMs (Fig. 2a, 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 SFN treatment in a concentration-dependent manner (Fig. 3a-c). To confirm these results *in vivo*, it was examined the effects of SFN 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 SFN treatment (Fig. 3d). Collectively, the results of this study showed that SFN 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.

Effects of SFN on PolyP-stimulated activation of NF-κB/ERK and production of IL-6/TNF-α: The NF-κB activation is required for proinflammatory responses and the three most important molecules involved in inflammatory signaling in the endothelial cells are NF-κB, TNF-α and IL-6[35-37]. Therefore, it was hypothesized that SFN might inhibit the expression or activity of these proinflammatory molecules. To investigate the potential effects of SFN on the production of the proinflammatory cytokines, IL-6 and TNF-α, HUVECs were incubated with SFN for 6 h after PolyP activation, followed by the measurement of IL-6 and TNF-α levels in the culture media via ELISA. The TNF-α and IL-6 levels showed an increase in PolyP-stimulated endothelial cells and these increases were significantly reduced $p<0.05$ by SFN (Fig. 4a, b), indicating that SFN can regulate the most important signals that induce proinflammatory responses in human endothelial cells. The activation of NF-κB and ERK1/2 is required for proinflammatory responses[36,38-39] 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 SFN. To confirm this hypothesis, the activation and expression levels of these proinflammatory molecules were measured with ELISA in PolyP-activated and SFN-treated HUVECs. The results showed that the increased protein expression levels of TNF-α and IL-6 (Fig. 4a, b) as well as the

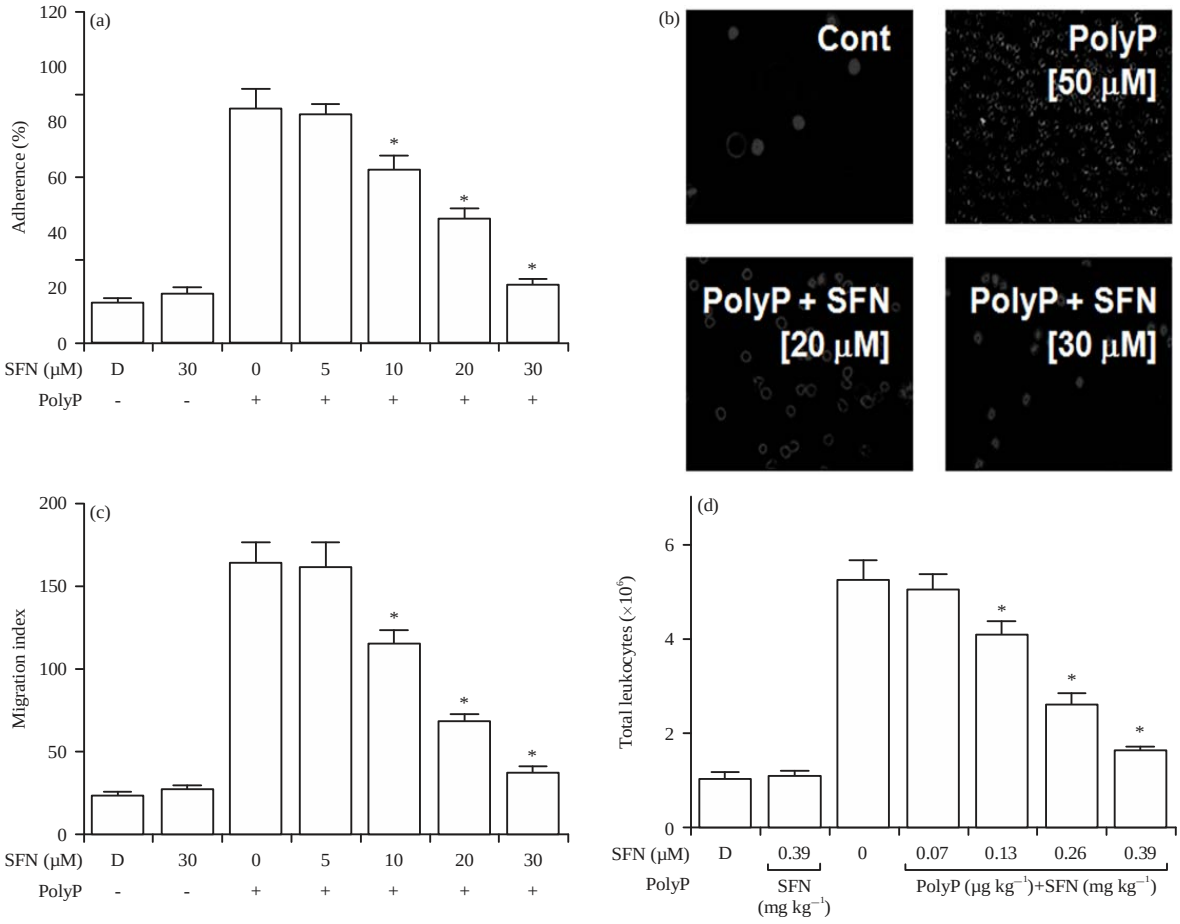

Fig. 3(a-d): Effects of SFN on PolyP-induced cell adhesion and TEM, (a) PolyP-mediated (50 M) adherence of neutrophils to HUVECs was analyzed after treating the cells with SFN, (b) Representative photomicrographs of neutrophil adhesion to HUVECs, (c) PolyP-mediated (50 M) migration of neutrophils through HUVEC monolayers was analyzed after treating the cells with SFN and (d) Effect of SFN on PolyP (3.5 μg mouse^{-1}, i.v.)-induced leukocyte migration in mice (expressed x10^6, n = 5)

The results are expressed as the Means±SD of three independent experiments, *p<0.05 vs. PolyP alone

increased activation of NF-κB and ERK1/2 (Fig. 4c, d) induced by PolyP were reduced by SFN. Therefore, these results suggested that SFN can control important vascular inflammatory signaling pathways by regulating the molecules involved.

Protective effect of SFN in PolyP-induced lethal model: Finally, it was hypothesized that SFN would prevent PolyP-mediated lethality in mice. To confirm this, the mice were administered SFN after PolyP injections. The results showed that treatment with a single dose of SFN (0.39 mg kg^{-1}, 12 h after PolyP injection) did not prevent PolyP-induced death. Thus, SFN was administered twice (once at 12 h and then 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). There was no additional survival effect above 0.39 mg kg^{-1} SFN. Moreover, when SFN was administrated orally, the survival effects of SFN were worse than those of intravenous injection (Fig. 5b, p<0.0001). If SFN was ingested as edible food, 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 as food, 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 SFN in foods is not absorbed. For a consumed compound to be utilized by the vascular system of the body, the four criteria (ADME), which represent the disposition of a pharmaceutical compound

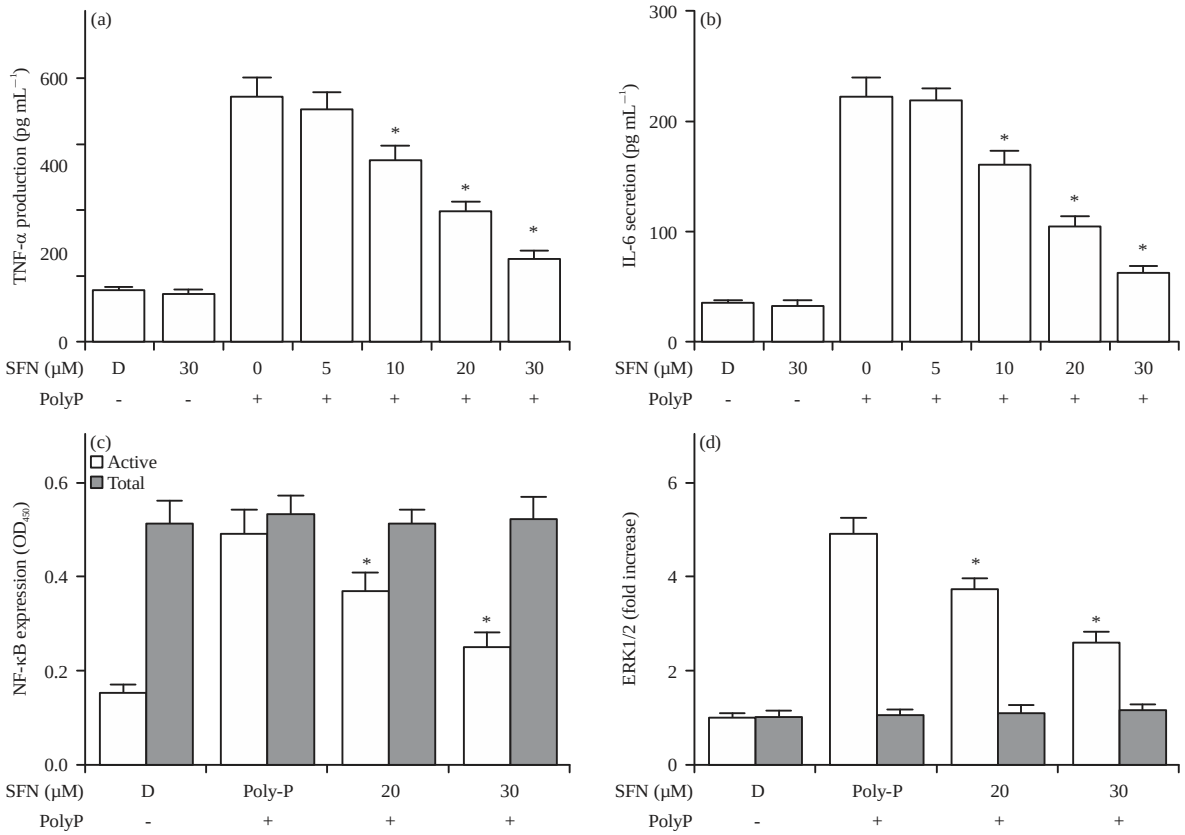

Fig. 4(a-d): Effects of SFN on PolyP-stimulated activation of NF-κB/ERK and production of IL-6/TNF-α. PolyP (50 M)-mediated production of (a) TNF-α, (b) IL-6 in HUVECs was analyzed after treatment of cells with the indicated concentrations of SFN for 6 h, (c) PolyP (50 M)-mediated activation of phospho-NF-κB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

*p<0.05 vs. PolyP alone

within an organism should be met. These four criteria influence the compound levels and 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[40], in this study examined the plasma levels of tissue damage markers. As shown in Fig. 5c-f, SFN reduced the polyP-induced increases in the plasma levels of ALT and AST (markers of hepatic injury, Fig. 5c) as well as creatinine and BUN (markers of renal injury, Fig. 5d, e). In addition, the levels of lactate dehydrogenase, which is a marker of tissue injury, were reduced by SFN in PolyP-injected mice (Fig. 5f).

Sepsis is defined as a Systematic Inflammatory Response Syndrome (SIRS) caused by infection Russell[41]. 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) Ballester *et al.*[42] and Angus and Wax[43]. Xigris (Eli Lilly) was approved in 2001 by the Food and Drug Administration (FDA) and in 2002 by the European Medicine Agency (EMA) for the treatment of severe sepsis and septic shock[44]. In October 2011, Xigris was withdrawn from the market owing to side effects and lack of beneficial effects on 28th day mortality in the PROWESS and septic shock (PROWESS-SHOCK) trials[45]. With the withdrawal of Xigris, there is no medicine to treat severe sepsis.

The molecular mechanisms underlying the anti-inflammatory effects of SFN on PolyP-mediated septic responses may be mediated by the suppression of PolyP-mediated hyperpermeability (Fig. 1a, b) via inhibition of p38 activation (Fig. 1c). Furthermore, the inhibitory mechanisms of SFN on the interaction of leukocytes with

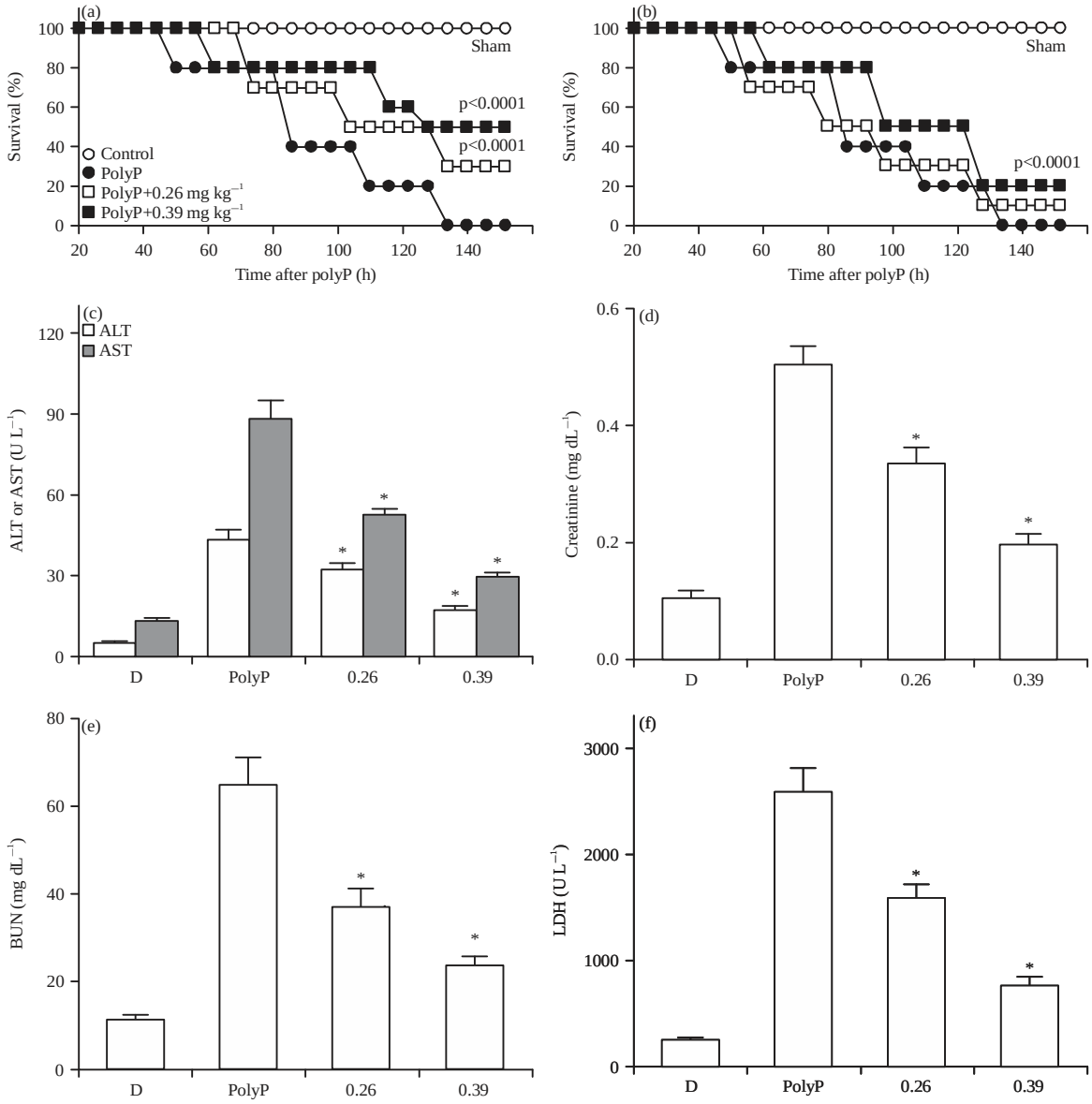

Fig. 5(a-f): Effects of SFN on PolyP-induced lethality and organ damage markers, (a, b) Male C57BL/6 mice (n = 20) were administered SFN at 0.26 (□) or 0.39 mg kg^{-1} (■) each, intravenously (a) or orally (b), 12 and 50 h after PolyP (3.5 μg mouse^{-1}, i.v.) injection. Animal survival was monitored every 6 h after PolyP injection for 132 h. PolyP-injected (●) and control mice (○) were administered 0.5% DMSO (n = 20). Kaplan-Meier survival analysis was used for the determination of overall survival rates vs. PolyP-treated mice, (c) The activity of hepatic injury markers AST and ALT, (d) The levels of renal injury markers creatinine, (e) BUN and (f) The tissue injury marker LDH

Levels were measured (n = 5) 72 h after PolyP injection, *p<0.05 vs. PolyP alone

endothelial cells are mediated by the inhibition of CAM expression, such as VCAM, ICAM and e-selectin (Fig. 2 and 3). The mechanisms underlying the anti-inflammatory effects of SFN involve the downregulation of inflammatory cytokine production (TNF-α and IL-6, Fig. 4a and b) and the activation of inflammatory transcriptional factors (NF-κB and ERK1/2, Fig. 4c, d).

CONCLUSION

The SFN was found to reduce PolyP-mediated septic lethality. The anti-septic mechanism of SFN may involve inhibiting PolyP-mediated hyperpermeability, suppressing the expression of inflammatory factors, inflammatory responses and alleviating tissue damage injury, which together raised

the survival rate of sepsis-induced mice. These findings suggest SFN could be considered a potential agent for the treatment of sepsis and other diseases in which PolyP is viewed as a therapeutic target.

SIGNIFICANCE STATEMENT

This study discovered the anti-septic effects of sulforaphane 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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