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Research Article
Inhibitory Effect of Sulforaphane on Secretory Group IIA Phospholipase A_2

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Abstract

Background and Objective: The expression of secretory group IIA phospholipase A2 (sPLA2-IIA) has been shown to be elevated in various inflammatory diseases and Lipopolysaccharide (LPS) up-regulates the expression of sPLA2-IIA in Human umbilical vein endothelial cells (HUVECs). Sulforaphane (SFN), a natural isothiocyanate present in cruciferous vegetables such as broccoli and cabbage, is effective in preventing carcinogenesis, diabetes and inflammatory responses. Here, SFN was examined for its effects on the expression and activity of $sPLA_2$-IIA in HUVECs and in mouse models of sepsis. **Materials and Methods:** After HUVECs were activated with LPS, cells were post-treated with SFN. *In vivo*, LPS-injected or Cecal ligation and puncture (CLP) operated mice were administrated SFN. Then, the effects of SFN on the activity and expression of sPLA2-IIA were determined by Enzyme-linked immunosorbent assay (ELISA). The effects of SFN on the activities of cytosolic phospholipase A2 (cPLA2) and Extracellular signal-regulated kinase (ERK)1/2 were monitored. Statistical relevance was determined by one-way analysis of variance (ANOVA). $p<0.05$ were considered to indicate significance **Results:** Post-treatment of cells or mice with SFN inhibited LPS- or CLP-induced expression and activity of $sPLA_2$-IIA. SFN also suppressed the activation of cPLA2 and ERK1/2 by LPS. **Conclusion:** It is concluded that, SFN inhibited LPS-mediated expression of $sPLA_2$-IIA by suppression of cPLA2 and ERK1/2.

Key words: Sulforaphane, HUVEC, secretory group IIA phospholipase A2, cecal ligation and puncture, lipopolysaccharide, inflammatory diseases

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

INTRODUCTION

A superfamily of Phospholipase A2 enzymes (PLA2) hydrolyzes the ester bond at the *sn*-2 position of phosphoglycerides to release a free fatty acid and lysophospholipids[1,2]. PLA2 consists of four individual groups, secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), Ca^{2+}-dependent PLA2 and lipoprotein-associated PLA2[1,2], which are grouped according to their characteristics that include molecular weight and Ca^{2+}-dependence. Among them, sPLA2 is activated in various inflammatory diseases, including rheumatoid arthritis, sepsis, bowel disease and respiratory distress syndrome[1,2]. Especially, patients suffering from sepsis, septic shock and polytrauma have shown the highest levels of secretory group IIA phospholipase A2 (sPLA2-IIA)[3,4]. In previous reports, sPLA2-IIA has been considered a regulator for a variety of biological mechanisms in mammalian cells involving coagulation, signal transduction, apoptosis, remodeling of cellular membranes and host defense[1,2].

It is known that Lipopolysaccharide (LPS), which is bacterial endotoxin, can cause lethal endotoxemia[5]. Endotoxins play crucial roles in activating innate immune responses and producing pro-inflammatory cytokines associated with vascular endothelial activation[6,7]. Lipid mediators contribute to the process of vascular inflammation, especially Prostaglandin E2 (PGE2), which is an inflammatory mediator or marker induced by bacterial infection[8]. PGE2 is derived from phospholipids through enzymatic reactions involving PLA2 and sPLA2-II2, which is the most abundant isoform of sPLA2[9]. Particularly, cPLA2α is recognized as an essential mediator of PGE2 activity, since the phosphorylated form of cPLA2α induced by extracellular signal-regulated kinase (ERK)1/2 produces arachidonic acid in response to inflammatory stimuli[10,11].

Sulforaphane (SFN) is an organosulfur compound that exhibits anti-cancer and anti-diabetic properties in experimental models and is found in cruciferous vegetables such as broccoli, Brussels sprouts and cabbage[12,13]. Heiss *et al.*[14] reported that SFN possesses anti-inflammatory properties, resulting in the down regulation of LPS-stimulated inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase (COX)-2 and Tumor necrosis factor (TNF)-a expression in RAW macrophages due to the inhibition of DNA binding of Nuclear factor-κB (NF-kB). Although some biological activities and pharmacological functions of SFN have been reported, the effects of SFN on the expression and activity levels of $sPLA_2$-IIA have not been previously determined. Since the induction of $sPLA_2$-IIA in endothelial cells is associated with inflammation, in this study, it was hypothesized that SFN will reduce the expression and activity levels of $sPLA_2$-IIA. In this study, it was aimed to investigate the effects of SFN on the expression and activity levels of $sPLA_2$-IIA and its potential as a useful drug candidate in the treatment of inflammatory diseases.

MATERIALS AND METHODS

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

Reagents: SFN, LPS (used at 100 ng mL^{-1}), ERK1/2 inhibitor (U0126) and $cPLA_2$a inhibitor (arachidonyl trifluoromethyl ketone, AACO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). $sPLA_2$-IIA was purchased from GenWay Biotech, Inc. (San Diego, CA, USA).

Cell culture: Primary Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science Inc. (Charles City, IA, USA) and maintained as described previously[15-18].

Animals and husbandry: Male C57BL/6 mice (6-7 weeks old, weighing 27 g), purchased from Orient Bio Inc. (Sungnam, Republic of Korea), were used after a 12 day acclimatization period. Mice were housed five per polycarbonate cage under controlled temperature (20-25°C) and humidity (40-45%) and a 12:12 h light: dark cycle. Mice received a normal rodent pellet diet and water *ad libitum* during acclimatization. All mice were treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University (IRB No. KNU 2016-54).

Cecal ligation and puncture (CLP): To induce sepsis, male mice were anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg kg^{-1}) and Rompun® (xylazine, 10 mg kg^{-1}). The CLP-induced sepsis model was prepared as previously described[19]. In brief, a 2 cm midline incision was made to expose the cecum and adjoining intestine. The cecum was tightly ligated with a 3.0 silk suture, 5.0 mm from the cecal tip and then punctured once using a 22-gauge needle to induce high grade sepsis[20]. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and returned to the peritoneal cavity. The laparotomy site was then sutured with 4.0 silk. In sham control

animals, the procedure was identical, except that the cecum was not ligated or punctured. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2016-54).

Enzyme-linked immunosorbent assay (ELISA) for $sPLA_2$-IIA expression: The level of $sPLA_2$-IIA protein in the cell culture medium was determined using a specific ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously[21] and following manufacturer's instructions. Primary HUVECs were activated with control serum-free media or 100 ng mL^{-1} LPS for 24 h, followed by incubating with the indicated concentrations of SFN for 6 h. For *in vitro* inhibitor studies, cells were incubated with U0126 (5 mM) or AACO (20 mM) for 2 h prior to LPS stimulation. For *in vivo* studies, LPS-injected mice (15 mg kg^{-1}, intraperitoneal) or CLP-operated mice were post-treated with SFN (0.26 or 0.39 mg kg^{-1}) for 6 h. After 2 days, plasma was prepared. Then, diluted medium or mouse plasma was added to each well of the ELISA plate and an acetylcholinesterase-$sPLA_2$-Fab' conjugate was added to each well after washing. The concentration of the analyte was measured by adding Ellman's reagent to each well and reading the product of the acetylcholinesterase-catalyzed reaction in an ELISA plate reader (Tecan, Mannedorf, Switzerland) at 412 nm. $sPLA_2$-IIA concentrations in the samples were calculated from a standard curve generated with recombinant $sPLA_2$-IIA.

Assay for the $sPLA_2$-IIA activity: The activity of $sPLA_2$-IIA was measured using 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino} dodecanoyl]-*sn*-3-phospho-ethanolamine (NBD-PE, Avanti Polar Lipids, Inc., Alabaster, AL, USA) as a substrate, as reported previously[22]. Reaction mixtures (total 100 mL) comprising 50 mM Tris–HCl (pH 8.0), 123 mM NBD-PE, 2 mM Ca^{2+} and the indicated amounts of $sPLA_2$-IIA were incubated for 30 min at 30°C in the presence or absence of the indicated concentrations of SFN.

Western blot analysis: Protein concentration was measured using a Bovine serum albumin (BSA) protein assay kit. Equal amounts of protein were electrophoresed on 10% Acrylamide-SDS-PAGE at 120 V in duplicates and were then transferred to nitrocellulose membranes at 200 mA for one hour. Membranes were blocked in Tris-buffered saline, pH 7.4 (TBS) with 0.1% Tween® 20 (TBS-T) containing 5% non-fat milk for one hour at room temperature and were then incubated with primary antibodies against phospho-ERK1/2 and ERK1/2 (1:10000), phospho-$cPLA_2$a and $cPLA_2$a (1:1000) overnight at 4°C. After washing with TBS-T, the blots were incubated with secondary antibodies for one hour at room temperature. Immunolabeling was detected by ECL (Millipore) and film exposure. Densitometry analysis was performed using the ImageJ Gel Analysis tool.

Statistical analysis: All experiments were performed independently at least three times. Values are expressed as Means±Standard Deviation (SD). The statistical significance of differences among test groups was evaluated with one-way analysis of variance (ANOVA) using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA). $p<0.05$ were considered to indicate significance differences[23].

RESULTS AND DISCUSSION

The effects of SFN on the expression and activity of sPLA2-IIA: The SFN was examined for its effects on the expression and activity of $sPLA_2$-IIA *in vitro* and *in vivo*. The concentration-dependence of the LPS-mediated expression of $sPLA_2$-IIA in primary human endothelial cells was determined. Analysis of the expression level of $sPLA_2$-IIA by the HUVECs in response to varying concentrations of LPS for 24 h indicated that the upregulation plateaued in cell culture supernatants at 100 ng mL^{-1} LPS. A similar effect of LPS was observed when endothelial cells were cultured in serum-free medium containing 0.2% BSA, excluding the possibility that the effect of LPS on $sPLA_2$-IIA expression is due to its interaction with some unknown factors in the serum. Based on these results, an LPS concentration of 100 ng mL^{-1} was used to stimulate endothelial cells in all experiments described below.

First, the effects of SFN on the expression and activity of $sPLA_2$-IIA induced by LPS in HUVECs were determined. The data show that post-treatment of SFN (at 10-30 mM) potently inhibited the expression (Fig. 1a) and activity of $sPLA_2$-IIA (Fig. 1b). Next, it was determined that the IC_{50} of SFN on TNF-a-induced $sPLA_2$-IIA activity was 4.14 mM. Thus, these results indicate that the expression and activity $sPLA_2$-IIA is significantly inhibited by SFN up to 73%, indicating that SFN has a significant effect $p<0.05$ on this enzyme.

The effect of SFN on sPLA2-IIA expression in LPS-induced endotoxemia and CLP-induced septic mice: To parallel the above indicated *in vitro* efficancy, SFN was evaluated *in vivo* for its inhibition of $sPLA_2$-IIA expression using mouse models

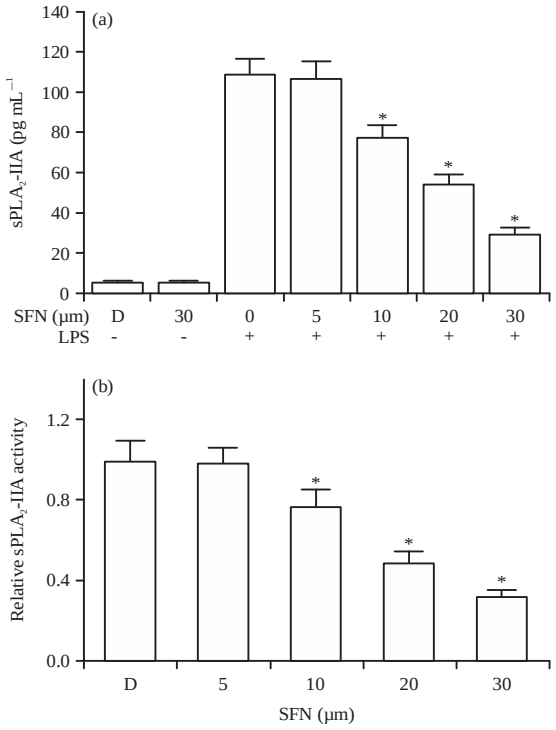

Fig. 1(a-b): Effect of SFN on the expression and activity of $sPLA_2$-IIA in endothelial cells. (a) Primary HUVECs were activated with serum-free media (as a control) or 100 ng mL^{-1} LPS for 24 h and were then incubated with the indicated concentrations of SFN for 6 h, before measuring the expression level of $sPLA_2$-IIA in culture medium. (b) The activity of $sPLA_2$-IIA was measured using NBD-PE as a substrate. Reaction mixtures (total 100 mL) comprising 50 mM Tris–HCl (pH 8.0), 123 mM NBD-PE, 2 mM Ca^{2+} and $sPLA_2$-IIA or $pPLA_2$ (approx. 2 mg) were incubated for 30 min at 30°C in the presence or absence of the indicated concentrations of SFN

All results are shown as Means±SD of three different experiments. D, 0.5% dimethyl sulfoxide vehicle control. *p<0.05 versus LPS only (a) or D (b)

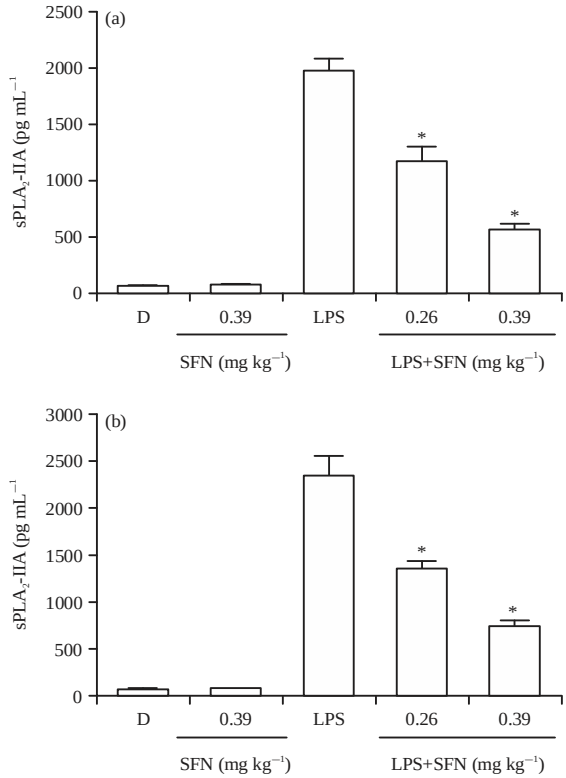

Fig. 2(a-b): Effect of SFN on the expression of $sPLA_2$-IIA in mouse. Male C57BL/6 mice (n = 5) were treated with SFN (0.26 or 0.39 mg kg^{-1}) after LPS injection (a) 15 mg kg^{-1}, intraperitoneal) or CLP surgery (b). After 2 days, mouse serum was prepared and the expression level of $sPLA_2$-IIA was measured

*p<0.05 versus LPS only (A) or CLP (B). All results are shown as Means±SD of three different experiments

of LPS-injected endotoxemia and CLP-induced sepsis. The presence of LPS, a bacterial endotoxin, ranks the highest among risk factors contributing to lethal endotoxemia[5]. Endotoxins are known to activate innate immune responses, resulting in the production of a vast spectrum of inflammatory cytokines[6,7]. These pro-inflammatory cytokines are known to induce vascular endothelial activation[24]. The CLP model of sepsis was used to determine the concentrations of serum $sPLA_2$-IIA present in severe vascular inflammatory diseases, because the CLP model closely resembles human sepsis[19]. At 24 h post-surgery, the animals manifested signs of sepsis, such as shivering, bristled hair and weakness. According to the results (Fig. 2), post-treatment with SFN markedly reduced $sPLA_2$-IIA expression in both LPS-injected and CLP-induced sepsis mice. The average circulating blood volume for mice is 72 mL kg^{-1} [25]. Noting that the average weight of our mice was 27 g and the average blood volume was 2 mL, the amount of SFN injected (0.26 or 0.39 mg kg^{-1}) yielded a maximum concentration of 20 or 30 mM, respectively, in the peripheral blood.

The inhibitory effects of POZ on LPS-induced activation of ERK1/2 and cPLA2α: Lipid mediators, such as PGE2, play a central role during vascular inflammatory processes and PGE2 is one of the central inflammatory markers and key mediators of inflammation induced by bacterial infection[8]. PGE2 is produced from phospholipids by a cascade of enzymatic reactions involving phospholipase A_2 (PLA_2) and $sPLA_2$-IIA is

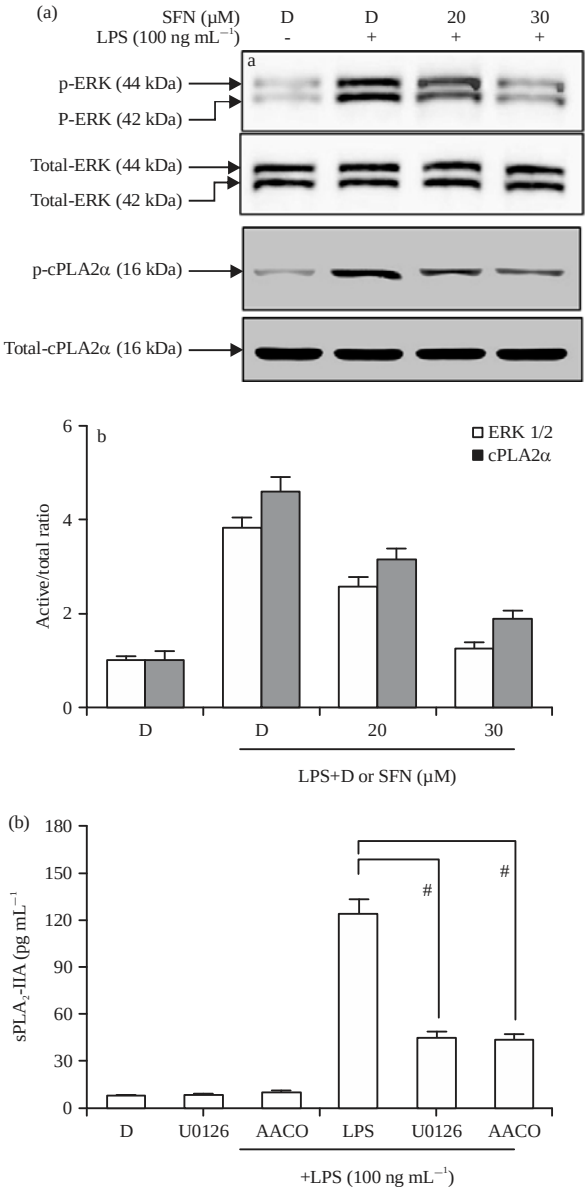

Fig. 3: Effect of SFN on the activation of ERK1/2 and cPLA$_2$a induced by LPS. (a) a: HUVECs were treated with LPS (100 ng mL^{-1}) for 24 h, followed by incubating with SFN (20 or 30 mM for 6 h). Expression of phosphorylated (p) and total cPLA$_2$a and ERK1/2 was assessed by western blotting. Illustrations indicate representative images from three independent experiments. b: The graphs show the densitometric intensities of phosphorylated ERK1/2 or phosphorylated cPLA$_2$a normalized to total levels. n = 3 blots. (b) Cells were preincubated with ERK1/2 inhibitor (U0126; 5 mM) or cPLA$_2$a inhibitor (AACO; 20 mM) for 2 h prior to LPS stimulation. The expression level of sPLA2-IIA in the culture medium was then measured

*p<0.05 versus LPS only (a) or #p<0.01 (b). All results are shown as Means±SD of three different experiments

the most abundant isoform of secreted PLA$_2$[9]. It is well established that cPLA$_2$a is essential for PGE2 production by supplying arachidonic acid for eicosanoid biosynthesis[26] and that the Mitogen-activated protein kinases (MAPKs), ERK1/2, contribute to phosphorylation of cPLA$_2$a in response to inflammatory stimuli[10]. Therefore, in order to test whether SFN could modulate the activation of cPLA$_2$a and ERK1/2 by LPS in human endothelial cells, HUVECs were activated with LPS and the activations of cPLA$_2$a and ERK1/2 were measured. The data show that SFN inhibits the activations of cPLA$_2$a and ERK1/2 that are induced by LPS, as shown in Fig. 3a.

Noting that SFN inhibited the activation of cPLA$_2$a and ERK1/2 by LPS treatment, next it was determined the role of ERK1/2 and cPLA$_2$a activation in LPS-mediated sPLA$_2$-IIA generation in human endothelial cells. Cells were pretreated with ERK1/2 inhibitor (U0126) or cPLA$_2$a inhibitor (AACO), followed by activation with LPS. The data show that treatment with U0126 or AACO suppressed the generation of sPLA$_2$-IIA by LPS (Fig. 3b). This indicates that LPS enhances the activation of ERK1/2 and cPLA$_2$a, which regulates the release of sPLA$_2$-IIA and the expression of sPLA$_2$-IIA is inhibited by SFN via suppression of ERK1/2 and cPLA$_2$a in human endothelial cells.

The sPLA$_2$-IIA seems to play a role in the initiation and propagation of vascular inflammation, such as severe sepsis, septic shock and polytrauma[3,4,27]. Supporting this, a high level of sPLA$_2$-IIA has been found in the sera of patients with inflammatory disorders[3,4]. However, the possibility that sPLA$_2$-IIA is only an inflammatory marker, rather than a contributor to inflammation, has not been ruled out because giving selective sPLA$_2$-IIA inhibitors to treat septic or rheumatoid arthritis patients failed to improve clinical outcomes[28,29]. Thus, a better clinical tool is needed to treat severe vascular inflammatory diseases. In this context, SFN might be an alternative candidate based on the inhibitory effects of SFN on the expression and activity of sPLA$_2$-IIA. This is supported by previous reports, which showed that hyper-permeability was found in sPLA$_2$-IIA transgenic mice[30] and that inflammatory chemokines and cell adhesion molecules could be induced directly by sPLA$_2$-IIA[31].

CONCLUSION

Post-treatment of cells or mice with SFN inhibited LPS- or CLP-induced expression and activity of sPLA$_2$-IIA and SFN suppressed the activation of cPLA2 and ERK1/2 by LPS. Therefore, the inhibitory effect of SFN on the expression and activity of sPLA$_2$-IIA may contribute to the anti-inflammatory effects of SFN in the endothelium via the inhibition of cPLA$_2$a and ERK1/2.

SIGNIFICANCE STATEMENTS

This study investigated the inhibitory effect of sulforaphane on sPLA$_2$-IIA *in vitro* and *in vivo*, which may be beneficial for development of inflammatory disease drug candidates. This study will help researchers to uncover critical areas of vascular inflammatory diseases that were not explored previously. Thus, a new theory of anti-inflammatory effects of natural compounds and possibly other drug combinations, will lead to the development of new sepsis treatments.

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