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# Inhibitory effect of exendin-4 on secretory group IIA phospholipase A2

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ABSTRACT

Exendin-4 (EX4), a glucagon-like peptide-1 receptor agonist, has been reported to attenuate myocardial ischemia and reperfusion injury and inflammatory or oxidative responses. The expression level of secretory group IIA phospholipase A2 (sPLA2-IIA) is elevated in inflammatory diseases. Lipopolysaccharide (LPS) upregulates the expression of sPLA2-IIA in human umbilical vein endothelial cells (HUVECs). Here, EX4 was examined for its effects on the expression and activity of sPLA2-IIA in HUVECs and mice. Pre-treatment of cells or mice with EX4 inhibited LPS-induced sPLA2-IIA expression and activity. Additionally, EX4 suppressed LPS-induced activation of cytosolic phospholipase A2 (cPLA2) and extracellular signal-regulated kinase (ERK) 1/2. Therefore, these results show that EX4 inhibited LPS-induced expression of sPLA2-IIA by suppressing cPLA2 and ERK 1/2.



## 1. Introduction

Phospholipases A2 (PLA2) are a superfamily of enzymes that hydrolyze the ester bond at the *sn*-2 position of phosphoglycerides to release a free fatty acid and lysophospholipids [1,2]. According to individual molecular weight and $Ca^{2+}$-dependence, the superfamily is divided into four groups: secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), $Ca^{2+}$-independent PLA2, and lipoprotein-associated PLA2 [1,2]. Although the exact biological functions of sPLA2-IIA are not completely understood, it is thought to be involved in a variety of biological processes in mammalian cells, such as coagulation, signal transduction, apoptosis, remodeling of cellular membranes, and host defense [3–5]. The most important risk factor of lethal endotoxemia is the presence of the bacterial endotoxin lipopolysaccharide (LPS) [6]. Endotoxins are known to activate innate immune responses and induce the production of a vast spectrum of pro-inflammatory cytokines [7,8]. These pro-inflammatory cytokines are known to trigger vascular endothelial activation [9]. It is well known that LPS and other inflammatory cytokines upregulate the transcription and protein expression of sPLA2-IIA in a variety of cells including macrophages [10], fibroblasts [11], endothelial cells [12], and astrocytes [13]. sPLA2 has been shown to have a significant connection with inflammatory diseases such as sepsis, bowel disease, acute pancreatitis, rheumatoid arthritis, bronchial asthma, and respiratory distress syndrome [14]. Further evidence for the role of sPLA2-IIA in inflammation comes from the discovery of large amounts of sPLA2-IIA in patients with severe inflammatory diseases such as sepsis, septic shock, and polytrauma [4,13,15–18]. Lipid mediators such as prostaglandin E2 (PGE2) play a central role during vascular inflammatory processes and PGE2 is one of the central inflammatory markers and key mediators of inflammation that are induced by bacterial infection [19,20]. PGE2 is produced from phospholipids by a cascade of enzymatic reactions involving PLA2, and sPLA2-IIA is the most abundant isoform of sPLA2 [1,2]. It is well established that cPLA2α is essential for PGE2 production because it supplies the arachidonic acid required for eicosanoid biosynthesis [21]. Mitogen-activated protein (MAP) kinase, extracellular signal-regulated kinase (ERK) 1/2, contributed to phosphorylation of cPLA2α in response to inflammatory stimuli [22].

Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone, which is secreted from L cells in the intestine in response to food intake; it has been shown to stimulate insulin secretion in response to oral nutrient intake [23]. GLP-1 receptor agonist exendin-4 (EX4)

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is a 39-residue peptide that was originally isolated from the salivary secretions of the lizard *Heloderma suspectum* (Gila monster) with a 53% amino acid similarity to GLP-1 [24]. Exendin 9-39 (EX9), a 36-residue fragment of EX4, is a GLP-1 receptor antagonist, which inhibits GLP-1-induced cAMP production and blocks GLP-1-induced insulin secretion [25]. While EX4 acts as an agonist of the GLP-1 receptor, EX9 acts as an antagonist of the GLP-1 receptor and has a binding affinity to the GLP-1 receptor that is similar to EX4 [26]. Several observations suggest that EX4 has a pleiotropic role in the cardiovascular system [27–29]. Furthermore, EX4 and GLP-1 analogues have been reported to have multiple cytoprotective effects—including the protection of endothelial cells against senescence mainly through antioxidant [30–32] and anti-inflammatory [32–34] processes. Since increase in GLP-1 levels has cardiovascular protective effect—probably through the anti-inflammatory activity of GLP-1 [35]—it is rational to hypothesize that the inflammatory response will be ameliorated by EX4 (i.e., GLP-1 analogue) treatment through the induction of GLP-1 receptor (GLP-1R) expression. However, the effects of EX4 or EX9 on the expression and activity levels of sPLA2-IIA have not yet been demonstrated. Since the induction of sPLA2-IIA expression in endothelial cells is related to inflammation, it is hypothesized that EX4 or EX9 reduce the expression and activity levels of sPLA2-IIA.

## 2. Materials and methods

### 2.1. Reagents

EX4, EX9, LPS (used at 100 ng/mL), ERK 1/2 inhibitor (U0126), and cPLA2α inhibitor (arachidonyl trifluoromethyl ketone; AACO) were purchased from Sigma (St. Louis, MO). sPLA2-IIA was purchased from GenWay Biotech, Inc (San Diego, CA).

### 2.2. Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described [36–39]. All experiments were performed using HUVECs at passages 3–5.

### 2.3. Animals and husbandry

Male C57BL/6 mice (6–7 weeks old; average weight, 20 g) were purchased from Orient Bio Co. (Sungnam, Republic of Korea) and used in this study after a 12-day acclimatization period. Five animals were housed per polycarbonate cage under controlled temperature (20–25 °C) and relative humidity (RH; 40–45%) with a 12:12-h light/dark cycle. Animals received a normal rodent pellet diet and water *ad libitum* during the acclimatization. All the animals were treated in accordance with the "Guidelines for the Care and Use of Laboratory Animals" issued by Kyungpook National University (KNU2012-13).

### 2.4. Cecal ligation and puncture (CLP)

For induction of sepsis, male mice were anesthetized with 2% isoflurane (Forane, JW pharmaceutical, South Korea) in oxygen delivered via a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA), first in a breathing chamber and then via a face-mask. They were allowed to breath spontaneously during the procedure. The CLP-induced sepsis model was prepared as previously described [36,40]. Briefly, a 2-cm midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated with a 3-0 silk suture 5.0 mm from the cecal tip and punctured once using a 22-gauge needle to induce sepsis [41]. It 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 cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to the start of the study (IRB No. KNU 2012-13).

### 2.5. ELISA for sPLA2-IIA expression

The level of sPLA2-IIA protein in the cell culture medium was determined using specific enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Arbor, MI) as described previously [42,43] according to the manufacturer's instructions. Primary HUVECs were preincubated with the indicated concentrations of EX4 or EX9 for 6 h. Alternatively, cells were preincubated with U0126 (5 μM) or AACO (20 μM) for 2 h. Cells were then incubated with serum-free media (as a control) or 100 ng/mL LPS for 24 h. For the *in vivo* experiments, LPS-treated (15 mg/kg, i.p.) or CLP-operated mice were treated with EX4 (167.5 or 251.2 ng/mouse, i.v.) or EX9 (134.8 or 202.2 ng/mouse, i.v.). After 2 days, plasma was prepared. Diluted medium or mouse plasma was added to each well of the plate. Subsequently, an acetylcholinesterase-sPLA2-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 measuring the product of the acetylcholinesterase-catalyzed reaction using an ELISA plate reader (Tecan, Mannedorf, Switzerland) at 412 nm sPLA2-IIA concentrations in the samples were calculated from a standard curve using recombinant sPLA2-IIA as a standard.

### 2.6. Assay for the sPLA2-IIA activity

The activity of sPLA2-IIA was measured using 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-3-phospho-ethanolamine (NBD-PE; AvantiPolar-lipid, Inc., Alabama) as a substrate, as reported previously [44]. Reaction mixtures (total 100 μL) consisting of 50 mM Tris–HCl (pH 8.0), 123 μM NBD-PE, 2 mM $Ca^{2+}$, and the indicated mounts of sPLA2-IIA were incubated for 30 min at 30 °C in the presence or absence of the indicated concentration of EX4 or EX9.

### 2.7. Western blot analysis

Protein concentration was measured using a bovine serum albumin (BSA) protein assay kit and loaded onto 10% acrylamide gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V, in duplicate, and then transferred to nitrocellulose membranes at 200 mA for 1 h. Membranes were then blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T; pH 7.4) containing 5% non-fat milk for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against phospho-ERK 1/2 and ERK 1/2 (1:10000), phospho-cPLA2a and cPLA2a (1:1000). After washing with TBS-T, blots were incubated with secondary antibodies for 1 h at room temperature. Immunolabeling was detected using enhanced chemiluminescence (ECL; Millipore). Densitometry analysis was performed using the ImageJ Gel Analysis tool.

### 2.8. Statistical analysis

Data are expressed as the means ± standard error mean of at least three independent experiments. Statistical significant differences between two groups were determined by using Student's *t*-test. The significance level was set at $p < 0.05$.

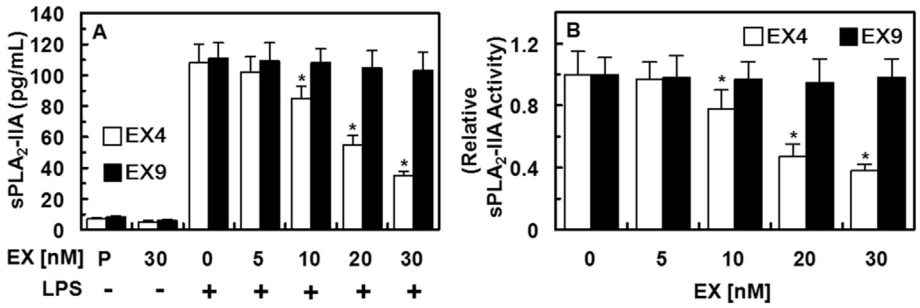


**Fig. 1.** Effect of EX4 or EX9 on the expression and activity of sPLA2-IIA in endothelial cells. (A) Primary HUVECs were preincubated with the indicated concentrations of EX4 or EX9 for 6 h. Then, cells were incubated with serum-free media (as a control) or 100 ng/mL LPS for 24 h before measuring the expression level of sPLA2-IIA in culture medium. (B) The activity of sPLA2-IIA was measured using NBD-PE as a substrate. Reaction mixtures (total 100 μL) comprising 20 μM Tris–HCl (pH 8.0), 123 μM NBD-PE, 2 mM $Ca^{2+}$, and sPLA2-IIA or pPLA2 (approx. 2 μg) were incubated for 30 min at 30 °C in the presence or absence of the indicated concentrations of EX4 or EX9. All results are shown as means ± SEM of three different experiments. P = PBS vehicle control. *$p < 0.05$ as compared to LPS only (A) or 0 (B).

## 3. Results and discussion

### 3.1. Effect of EX4 on the expression and activity of sPLA2-IIA in the LPS activated HUVECs

In primary HUVECs, analysis of the expression level of sPLA2-IIA in response to varying concentrations of LPS for 24 h indicated that the induction level reaches a plateau in cell culture supernatants at 100 ng/mL LPS (data not shown). A similar result was obtained when HUVECs were cultured in serum-free medium containing 0.2% BSA; this excluded the possibility that the effect of LPS on sPLA2-IIA expression was due to its interference with factors related to the serum content of the culture medium. Based on these results, an LPS concentration of 100 ng/mL was used to stimulate endothelial cells in the experiments described below.

First, it was investigated whether or not EX4 or EX9 could modulate LPS-induced sPLA2-IIA expression. It was found that, at 10–30 nM, EX4 but not EX9 could potently inhibit the expression of sPLA2-IIA in LPS-stimulated HUVECs (Fig. 1A). Furthermore, EX4 dose-dependently inhibited sPLA2-IIA activity with 50% inhibition ($ID_{50}$) at approximately 22.6 nM (Fig. 1B). Therefore, EX4 inhibited both the expression and activity sPLA2-IIA in LPS-stimulated HUVECs, indicating that EX4 had a significant effect on this enzyme.

### 3.2. Effect of EX4 on the expression of sPLA2-IIA in mice with LPS-induced endotoxemia or CLP-induced sepsis

To confirm the inhibitory effect of EX4 on sPLA2-IIA *in vivo*, mice with LPS-induced endotoxemia or CLP-induced sepsis were used. The CLP-induced sepsis model closely resembles human sepsis [45]. At 24 h after the operation, animals manifested signs of sepsis such as shivering, bristled hair, and weakness. The results are shown in Fig. 2. Treatment with EX4 markedly reduced sPLA2-IIA expression in both endotoxemia and sepsis mouse models. Assuming that the average weight of a mouse was 20 g and the average blood volume was 2 mL, the amount of EX4 (167.5 or 251.2 ng/mouse, i.v.) or EX9 (134.8 or 202.2 ng/mouse, i.v.) was equivalent to 20 or 30 nM in peripheral blood.

### 3.3. EX4 suppress the activation of ERK 1/2 and cPLA2α induced by LPS

Next, we determined the effects of EX4 on the activation of cPLA2α and ERK 1/2 in LPS-stimulated HUVECs. The data showed that LPS stimulated the phosphorylation of cPLA2α and ERK 1/2, and EX4 attenuated the LPS-induced phosphorylation of cPLA2α and ERK 1/2 (Fig. 3A). Since our results indicated that the activation of cPLA2α and ERK 1/2 was amplified by LPS and inhibited by EX4, we then evaluated the role of ERK 1/2 and cPLA2α activation in LPS-mediated sPLA2-IIA generation in HUVECs. Cells were pretreated with an inhibitor of ERK 1/2 (U0126) or cPLA2α (AACO) and then exposed to LPS. As shown in Fig. 3B, U0126 and AACO treatment decreased LPS-induced sPLA2-IIA generation. These results implied that LPS-induced activation of ERK 1/2 and cPLA2α regulates sPLA2-IIA release in HUVECs, and that EX4 inhibit LPS-mediated expression of sPLA2-IIA by suppressing ERK 1/2 and cPLA2α.

The involvement of sPLA2-IIA in inflammatory diseases in humans such as sepsis, septic shock, and polytrauma is well documented, and its expression is well correlated with the severity of inflammatory diseases [4,13,15–18]. The expression level of sPLA2-IIA is markedly increased by pro-inflammatory mediators and downregulated by anti-inflammatory cytokines in a variety of

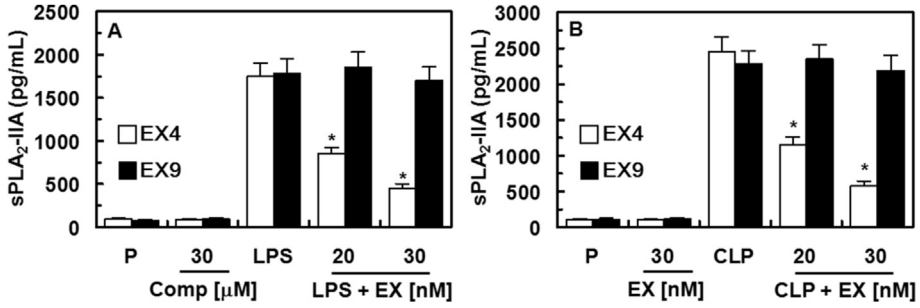


**Fig. 2.** Effect of EX4 or EX9 on the expression of sPLA2-IIA in mice. Male C57BL/6 mice (n = 5) were treated with EX4 (167.5 or 251.2 ng/mouse, i.v.) or EX9 (134.8 or 202.2 ng/mouse, i.v.) before LPS injection (A, 15 mg/kg, i.p.) or CLP surgery (B). After 2 days, mouse serum was prepared, and the expression level of sPLA2-IIA was measured. *$p < 0.05$ as compared to LPS only (A) or CLP (B).

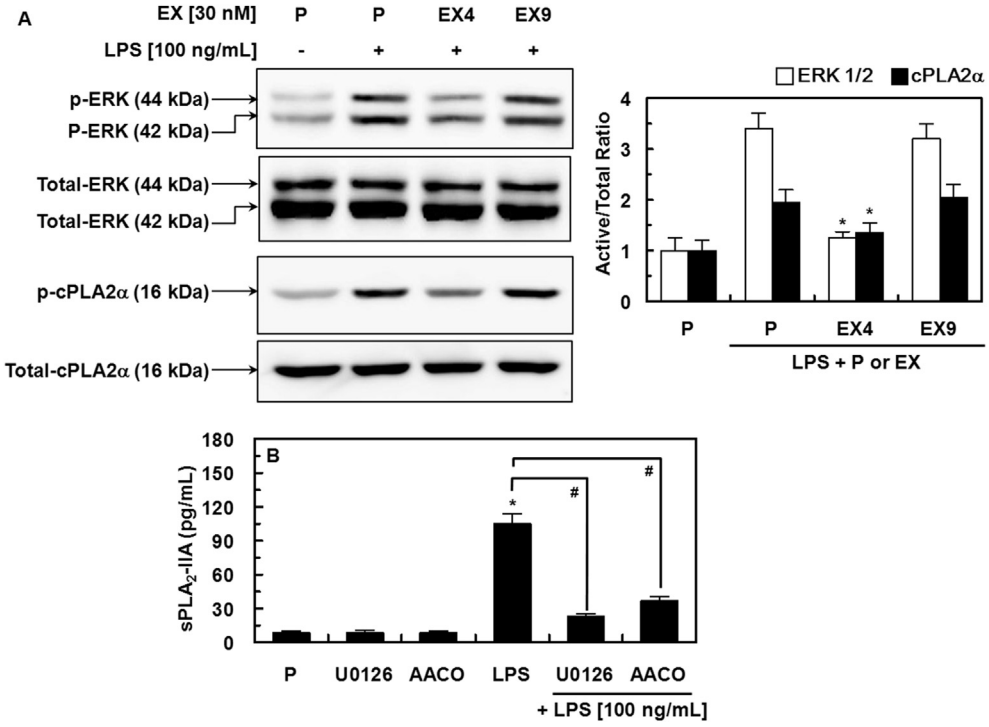


**Fig. 3.** Effect of EX4 or EX9 on LPS-induced activation of ERK 1/2 and cPLA2α. (A) (Left) HUVECs were incubated with EX4 or EX9 (20 μM for 6 h), followed by treatment with LPS (100 ng/mL) for 24 h. Expression of phosphorylated (p) and total cPLA2α and ERK 1/2 was assessed by western blotting. Illustrations indicate representative images from three independent experiments. (Right) The graphs show the densitometric intensities of phosphorylated ERK 1/2 or cPLA2α normalized to total levels. n = 3 blots. (B) As shown in Fig. 2A except that cells were preincubated with ERK 1/2 inhibitor (U0126; 5 μM) or cPLA2α inhibitor (AACO; 20 μM) for 2 h $^{*}p < 0.05$ as compared to LPS only (A) or $^{\#}p < 0.05$ (B).

mammalian cells and tissues [13,17,18]. Therefore, sPLA2-IIA expression is thought to be associated with the initiation and exacerbation of inflammatory reactions. In support of this, inflammatory diseases have been shown to be ameliorated by sPLA2-IIA inhibitors [46–48], and in turn, purified sPLA2-IIA aggravates inflammatory responses when injected into inflamed tissues [49]. Thus, sPLA2-IIA is implicated in the pathophysiology of several inflammatory diseases. Although specific inhibitors are able to reduce the abnormal production of sPLA2-IIA, they failed to improve the clinical outcome in patients with severe sepsis or rheumatoid arthritis [47,50]. Therefore, an improved approach is needed in order to cure severe inflammatory diseases. EX4 is a candidate inhibitor of sPLA2-IIA expression. This is supported by the discovery that sPLA2-IIA transgenic mice develop hyper permeability [51], and sPLA2-IIA directly induces the expression of chemokines and cell adhesion molecules in the vascular endothelium [52]. From this perspective, EX4 is of special interest since in this study EX4 inhibited the expression and activity of sPLA2-IIA.

In summary, the results presented in this study showed that EX4 can inhibit sPLA2-IIA expression and activity in cultured endothelial cells and mice through the inhibition of cPLA2α and ERK 1/2.

## Conflict of interest

The authors have no conflict of interest to declare.

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## Transparency document

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