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Inhibitory Effect of FXa on Secretory Group IIA Phospholipase A_2

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Abstract—It is well known that the expression level of secretory group IIA phospholipase A_2 (sPLA$_2$-IIA) is elevated in inflammatory diseases and lipopolysaccharide (LPS) upregulates the expression of sPLA$_2$-IIA in human umbilical vein endothelial cells (HUVECs). Activated factor X (FXa) is an important enzyme in the coagulation cascade responsible for thrombin generation, and it influences cell signaling in various cell types by activating protease-activated receptors (PARs). Here, FX or FXa was examined for its effects on the expression and activity of sPLA$_2$-IIA in HUVECs and mouse. Prior treatment of cells or mouse with FXa inhibited LPS-induced expression and activity of sPLA$_2$-IIA *via* interacting with FXa receptor (effective cell protease receptor-1, EPR-1). And FXa suppressed the activation of cytosolic phospholipase A2 (cPLA2) and extracellular signal-regulated kinase (ERK) 1/2 by LPS. Therefore, these results suggest that FXa may inhibit LPS-mediated expression of sPLA$_2$-IIA by suppression of cPLA2 and ERK 1/2.

KEY WORDS: FXa; HUVEC; sPLA$_2$-IIA; inflammation.

INTRODUCTION

Coagulation, i.e., formation of fibrin, is initiated by complex formation of tissue factor with the protease factor VIIa and is propagated by proteolytic activity of two other proteases, activated factor X (FXa) and thrombin [1]. The serine protease zymogen factor X (FX) is converted to FXa on binding to the binary complex of the cell surface receptor tissue factor (TF) with its protease ligand factor VIIa (VIIa) or by the intrinsic pathway [2]. The transient TF: VIIa:FXa ternary complex is the target for physiological inhibitory control by TF pathway inhibitor [3]. On dissociation from TF: VIIa and association with its cofactor FVa, FXa proteolytically converts prothrombin to thrombin, which in turn leads to fibrin formation, fibrin deposition, and thrombus formation [4]. Coagulation proteases, such as FXa and thrombin, can induce multiple cellular effects *via* activation of protease-activated receptors (PARs) [5, 6]. PARs are the family of G-protein-coupled receptors (GPCR) that are activated by proteolytic cleavage. PAR-1 is activated by various proteases, including FXa and thrombin, whereas PAR-2 is activated by the TF:FVIIa complex, FXa, and other proteases. It has been demonstrated that coagulation protease-dependent activation of PARs contributes to inflammation in many vascular disorders [5, 6].

There are at least 20 distinct mammalian phospholipases A2 (PLA2) enzymes (of which 18 occur in humans) that catalyze the hydrolysis of the *sn*-2 ester bond (middle) of a phospholipid to generate a fatty acid and a lysophospholipid [7, 8]. PLA2 enzymes can be broadly classified into three major classes on the basis of their requirements for calcium, the mechanism of their catalytic action, their molecular weight and also sequence homology: secretory PLA_2 (sPLA$_2$), cytosolic PLA_2, Ca^{2+}-independent PLA_2 and lipoprotein associated PLA_2 [7, 8]. The secretory enzymes, sPLA$_2$ groups IB, IIA, IID, IIE, IIF, V, X, and XIIA, are all approximately 14 kDa and all possess a catalytic histidine residue that functions as a general base to activate a water molecule for hydrolysis of the ester bond [7, 8]. sPLA$_2$ group IIA (sPLA$_2$-IIA) was implicated early following the observation that large

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amounts of $sPLA_2$-IIA are present in the synovial fluid of rheumatoid arthritis patients and in platelets in the joint [9, 10]. Additionally, massively elevated levels of $sPLA_2$-IIA were found in the serum of patients with septic shock acute pancreatitis and peritonitis and that $sPLA_2$-IIA gene expression is upregulated by inflammatory cytokines and sub-nanomolar levels of bacterial lipopolysaccharide (LPS) [11–16]. Together, these findings provided circumstantial evidence for a causative role for this enzyme in these diseases. However, the effects of FXa on the expression and activity levels of $sPLA_2$-IIA have not been studied yet. Since the induction of $sPLA_2$-IIA in endothelial cells is related with inflammation, in this study, it is hypothesized that FXa might reduce the expression and activity levels of $sPLA_2$-IIA.

MATERIALS AND METHODS

Regents

FX and FXa were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). LPS (used at 100 ng/mL), extracellular signal-regulated kinase (ERK) 1/2 inhibitor (U0126), and cPLA2α inhibitor (arachidonyl trifluoromethyl ketone, AACO) were purchased from Sigma (St. Louis, MO, USA). $sPLA_2$-IIA was purchased from GenWay Biotech, Inc (San Diego, CA, USA). FXa receptor (effective cell protease receptor-1, EPR-1) antibody was obtained from Alpha Diagnostica Int. Inc. (San Antonia, TX, USA).

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science Inc. (Charles City, IA) and maintained as described as before [17]. All experiments were performed using HUVEC cells passaged three or five times.

Animals and Husbandry

Male C57BL/6 mice (6–7 weeks old; average weight, 20 g) purchased from Orient Bio Co. (Sungnam, Republic of Korea) were used in this study after a 12-day acclimatization period. The animals were housed 5 per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40–45 % RH) and 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 (IRB No. KNU2012-13).

Cecal Ligation and Puncture

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 facemask. They were allowed to breath spontaneously during the procedure. The cecal ligation and puncture (CLP)-induced sepsis model was prepared as previously described [17, 18]. In brief, 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 at 5.0 mm from the cecal tip and punctured once using a 22-gauge needle for induction of high grade sepsis [19]. 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 conduct of the study (IRB No. KNU 2012–13).

ELISA for $sPLA_2$-IIA Expression

The level of $sPLA_2$-IIA protein in the cell culture medium was determined by using specific ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously [20, 21] according to the manufacturer's instruction. Primary HUVECs were preincubated with indicated concentrations of FX or FXa for 6 h with or without incubation with anti-EPR-1 antibodies for 1 h. Or, cells were preincubated with U0126 (5 μM) or AACO (20 μM) for 2 h. Then, cells were incubated with control serum-free media or 100 ng/mL LPS for 24 h. Or, LPS injection (15 mg/kg, i.p.) or CLP-operated mice were treated with FX (1.2 or 2.4 μg/mouse) or FXa (0.9 or 1.8 μg/mouse). After 2 days, plasma was prepared. Then, diluted medium or mouse plasma was added to each well of the plate. Then, 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 using recombinant $sPLA_2$-IIA as a standard.

Assay for the sPLA$_2$-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, USA) as a substrate, as reported previously [22]. Reaction mixtures (total 100 μL) comprising 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 concentrations of FX or FXa.

Western Blot Analysis

Protein concentration was measured by using a bovine serum albumin (BSA) protein assay kit and loaded on the 10 % Acrylamide-SDS-PAGE at 120 V in duplicates for electrophoresis and then transferred to nitrocellulose membranes at 200 mA for 1 h. Membranes were then blocked in Tris-buffered saline, pH 7.4 (TBS) with 0.1 % Tween 20 (TBS-T) containing 5 % nonfat milk for 1 h at room temperature and then incubated with primary antibodies against phospho-ERK1/2, ERK1/2, phospho-cPLA2a, cPLA2a, Cyclooxygenases-2 (COX-2), microsomal PGE synthase-1 (mPGES-1), or β-actin overnight at 4 °C. After washing with TBS-T, blots were incubated with secondary antibodies for 1 h at room temperature. Immunolabeling was detected by ECL (Millipore).

Statistical Analysis

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

RESULTS AND DISCUSSION

In this study, the influence of factor X (FX) or activated factor X (FXa) on the expression and activity of sPLA$_2$-IIA were examined *in vitro* and *in vivo*.

Effect of FX or FXa on the Expression and Activity of sPLA$_2$-IIA in the LPS-Activated HUVECs

It is well known that LPS and other inflammatory cytokines upregulate the transcription of sPLA$_2$-IIA and its protein level in a variety of cells including macrophage [23], fibroblast [24], endothelial cells [25], and astrocytes [15]. Analysis of the expression level of sPLA$_2$-IIA by primary HUVECs in response to varying concentrations of LPS for 24 h indicated that the induction level reaches 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, excluding the possibility that the effect of LPS on sPLA$_2$-IIA expression was due to its interference with factor related with to serum of cell culture medium. Based on these results, LPS concentration at 100 ng/mL was used to stimulate endothelial for the further experiments described below.

First, it is investigated whether FX or FXa could modulate LPS-induced sPLA$_2$-IIA expression and found that FXa, but not FX, at 5–20 μM potently inhibits the expression of sPLA$_2$-IIA in LPS-stimulated HUVECs (Fig. 1a). Furthermore, FXa inhibited concentration-dependently sPLA2-IIA activity with 50 % inhibition (ID_{50}) at approximately 13.08 nM (Fig. 1b).

The FX/FXa receptor on endothelial cells was identified as the effector cell protease receptor-1 (EPR-1) which is responsible for the cellular effect of the protease in endothelial cells (37). To confirm the inhibitory effects of FXa on the expression and activity of sPLA$_2$-IIA in the LPS-activated HUVECs, anti-EPR-1 antibodies were pre-incubated, followed by cells that were activated with LPS. As shown in Fig. 1c, d, anti-EPR-1 antibodies abolished the inhibitory effects of FXa on the expression and activity of sPLA$_2$-IIA. Therefore, FXa inhibited both the expression and activity sPLA$_2$-IIA in the LPS-activated HUVECs, suggesting a significance role of FXa on this enzyme.

Effect of FX or FXa on the Expression of sPLA$_2$-IIA in LPS-Injected or CLP-Induced Sepsis Mouse

To confirm the inhibitory effects of FXa on sPLA$_2$-IIA *in vivo*, FX or FXa were evaluated for their inhibition of sPLA$_2$-IIA expression using a model of LPS-injected endotoxemia or CLP-induced sepsis mouse. Presence of LPS, a bacterial endotoxin, ranks the highest among the risk factors contributing to lethal endotoxemia [26]. Endotoxins are known to activate innate immune responses, resulting in the production of a vast spectrum of inflammatory cytokines [27, 28]. These pro-inflammatory cytokines are known to trigger vascular endothelial activation [29]. And, 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 [30]. At 24 h after the operation, animals manifested signs of sepsis, such as shivering, bristled

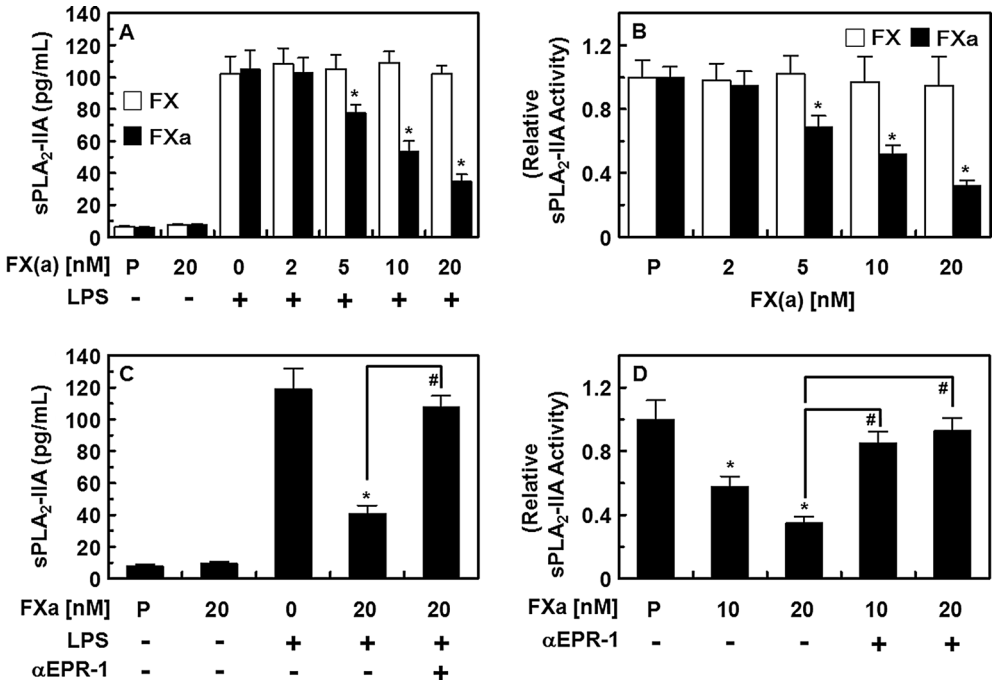

Fig. 1. Effect of FX or FXa on the expression and activity of $sPLA_2$-IIA in endothelial cells. **a** Primary HUVECs were preincubated with indicated concentrations of FX or FXa for 6 h. Then, cells were incubated with control serum-free media or 100 ng/mL LPS for 24 h followed by measuring the expression level of $sPLA_2$-IIA in culture medium as described under "Materials and Methods." **b** The activity of $sPLA_2$-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 $sPLA_2$-IIA or pPLA2 (approx. 2 μg) were incubated for 30 min at 30 °C in the presence or absence of indicated concentrations of FX or FXa. **c**, **d** The same as **a**, **b** except that cells were preincubated with anti-EPR-1 antibodies for 1 h. All results are shown as means ± SEM of three different experiments. *P* = PBS is the vehicle control. $*p < 0.05$ as compared to LPS only **a**, **c** or P **b**, **d** and $^{\#}p < 0.05$.

hair, and weakness. In the results (Fig. 2), treatment with FXa showed a marked reduction in the $sPLA_2$-IIA expression in both LPS-injected or CLP-induced sepsis mouse. Assuming that the average weight of a mouse was 20 g and average blood volume was 2 mL, the amount of FX (1.2 or 2.4 μg/mouse, i.v.) or FXa (0.9 or 1.8 μg/mouse, i.v.) was equivalent to 10 or 20 μM in peripheral blood.

FXa Suppress the Activation of ERK1/2 and cPLA2α Induced by LPS

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 induced by infection [31–33]. PGE2 is produced from phospholipids by a cascade of

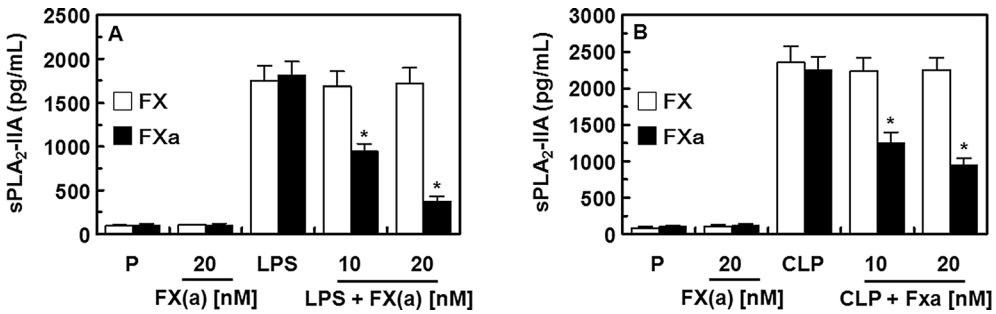

Fig. 2. Effect of FX or FXa on the expression of $sPLA_2$-IIA in mouse. Male C57BL/6 mice ($n = 5$) were administered with FX (1.2 or 2.4 μg/mouse, i.v.) or FXa (0.9 or 1.8 μg/mouse, i.v.) before LPS injection (**a**, 15 mg/kg, i.p.) or CLP-surgery (**b**). After 2 days, mouse serum was prepared and the expression level of $sPLA_2$-IIA was measured as described under "Materials and Methods." *P* = PBS is the vehicle control. $*p < 0.05$ as compared to LPS only (**a**) or CLP (**b**).

enzymatic reactions involving phospholipase A2 (PLA2), and $sPLA_2$-IIA is the most abundant isoform of secreted PLA2 [7, 8]. It is well established that cPLA2α is essential for PGE2 production by supplying arachidonic acid for eicosanoid biosynthesis [34, 35] and MAP kinase, ERK 1/2, contributed to phosphorylation of cPLA2α in response to inflammatory stimuli [34, 36]. Therefore, we determined the effects of FX or FXa on the activation of cPLA2α and ERK 1/2 in LPS-activated HUVECs. The data showed that LPS stimulated the phosphorylation of cPLA2α and ERK 1/2, and FXa attenuated-LPS induced the phosphorylation of cPLA2α and ERK 1/2 (Fig. 3a).

Since our results indicated that LPS amplified and FXa inhibited the activation of cPLA2α and ERK 1/2, we then evaluated the role of ERK 1/2 and cPLA2α activation in LPS-mediated $sPLA_2$-IIA generation in HUVECs. Cells were pretreated with an inhibitor of ERK 1/2 inhibitor (U0126) or cPLA2α (AACO) and then exposed to LPS. As shown in Fig. 3b, U0126 and AACO treatment did decrease the $sPLA_2$-IIA generation. These results imply that activation of ERK 1/2 and cPLA2α by LPS regulates $sPLA_2$-IIA release in HUVECs and FXa might inhibit LPS-mediated expression of $sPLA_2$-IIA by suppression of ERK 1/2 and cPLA2α.

FXa Prevents LPS-Induced Activation of COX2 and mPGES-1

COX-2 is a crucial enzyme for the production of PGE2 during inflammation and it has been shown to be dependent upon the activation of cPLA2α *via* a feedback mechanism as cPLA2α provides the substrate (arachidonic acid) for COX-2 [37]. Arachidonic acid is further metabolized by COX-2 into prostaglandin G2 (PGG2), which

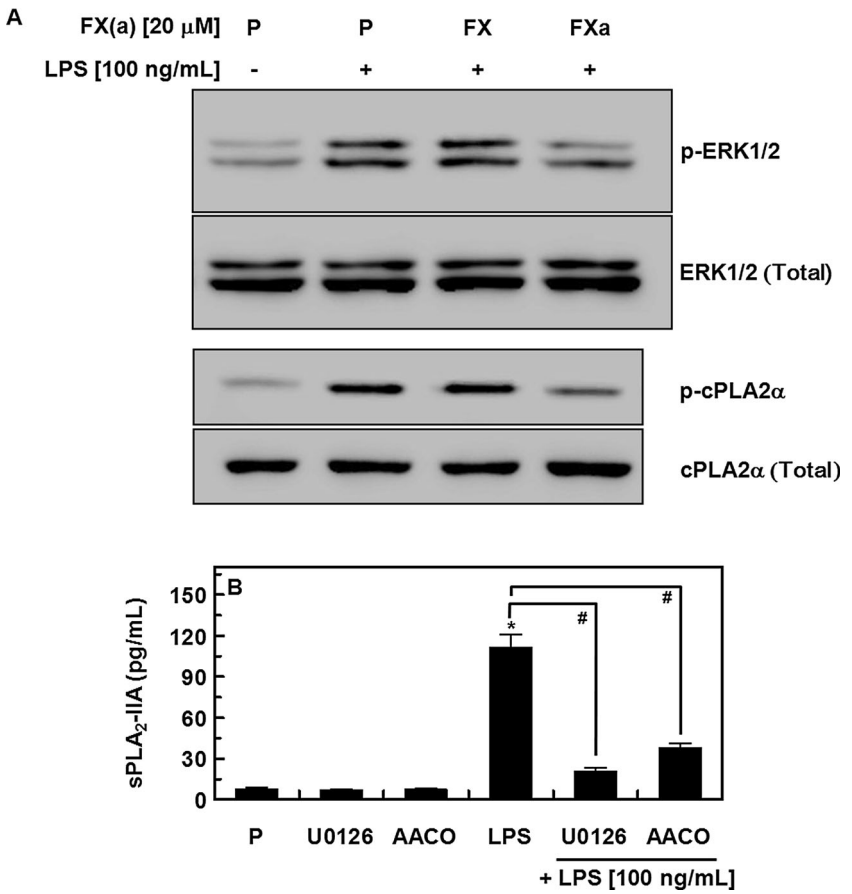

Fig. 3. Effect of FX or FXa on the activation of ERK1/2 and cPLA2α induced by LPS. **a** HUVECs were incubated with FX or FXa (20 μM for 6 h), followed by LPS (100 ng/mL) for 24 h. Expression of phosphorylated (*p*) and total cPLA2α and ERK1/2 was assessed by Western blotting as described under "Materials and Methods." *P*=PBS is the vehicle control. Illustrations indicate representative images from three independent experiments. **b** The same as Fig. 1a 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 P (PBS) and $^{\#}p<0.05$.

upon conversion to PGH2 serves as the substrate for specific prostaglandin synthases such as mPGES-1 [38]. Thus, inhibition of COX-2 in combination with downstream mPGES-1 appears to be responsible for the decreased biosynthesis of PGE2 and the alleviation of inflammation. Therefore, we investigated whether the expression of COX-2 and mPGES-1 is regulated by FXa. Data showed that the COX-2 enzyme was not expressed under basal conditions in HUVECs but was induced by LPS stimulation and was downregulated in the presence of FXa (Fig. 4a). And COX-2 dependent final enzyme in this cascade, mPGES-1, was found to be constitutively expressed and was upregulated upon LPS treatment; the change was significantly prevented by FXa (Fig. 4a). Our results demonstrate that LPS activates the phosphorylation of ERK 1/2, the accumulation of ERK 1/2 levels modulates the downstream activates of cPLA2α (Fig. 3a), and cPLA2α regulates COX-2 and mPGES-1 enzyme activations which lead to the production of $sPLA_2$-IIA (Fig. 4b). And FXa significantly decreased the phosphorylation of ERK 1/2 and cPLA2α, and the expression of COX-2 and mPGES-1 enzyme, confirming the inhibitory effects of FXa in the $sPLA_2$-IIA synthesis induced by LPS (Fig. 4b).

The involvement of $sPLA_2$-IIA in inflammatory diseases in humans is well documented such as sepsis, septic shock, and polytrauma and it is well correlated with the severity of inflammation diseases [11–16]. The expression level of $sPLA_2$-IIA is markedly induced by pro-inflammatory mediators and downregulated by anti-inflammatory cytokines in a variety of cells and tissues in mammalians [14–16]. Therefore, the $sPLA_2$-IIA is thought to associate with the initiation and multiplication of

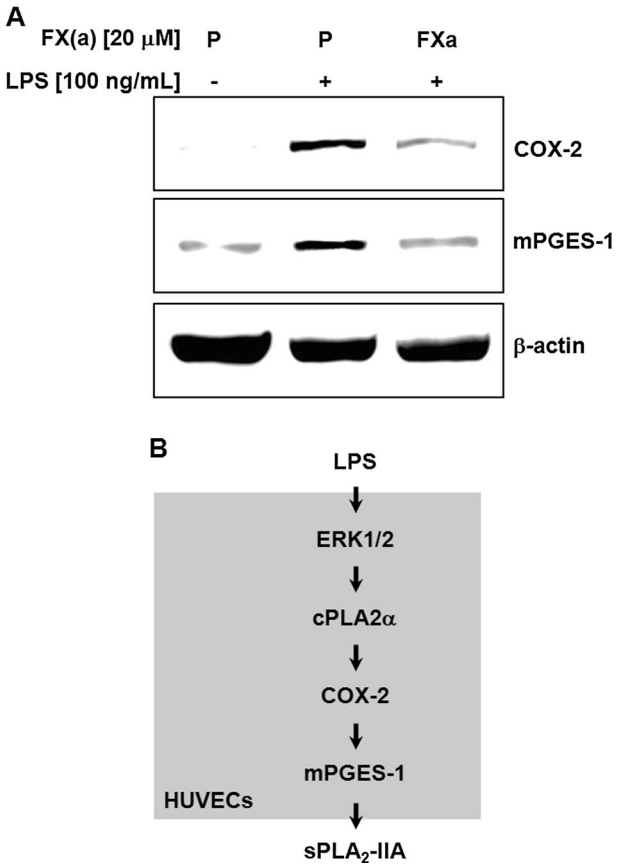

Fig. 4. Effect of FXa on LPS-induced expression of COX-2 and mPGES-1. **a** HUVECs were incubated with FXa (20 μM for 6 h), followed by with LPS (100 ng/mL) for 24 h. Expression of COX-2 and mPGES-1 was assessed by Western blotting as described under "Materials and Methods." *P*=PBS is the vehicle control. Illustrations indicate representative images from three independent experiments. **b** Possible molecular mechanism involved in LPS-induced $sPLA_2$-IIA production in HUVECs. LPS activates the phosphorylation of ERK 1/2. The accumulation of ERK 1/2 levels modulates the downstream activates of cPLA2a, cPLA2a regulates COX-2 and mPGES-1 enzyme activations which lead to the production of $sPLA_2$-IIA. *LPS*, lipopolysaccharide; *cPLA2α*, cytosolic phospholipase A2α; *COX-2*, cyclooxygenase-2; *mPGES-1*, microsomal PGE synthase-1; *$sPLA_2$-IIA*, secretory phospholipase A_2-IIA.

inflammatory reactions. In supporting this, the inflammatory diseases are attenuated by sPLA$_2$-IIA inhibitors [39–41], and in turn, purified sPLA$_2$-IIA aggravates these responses when injected into inflamed tissues [42]. Thus, sPLA$_2$-IIA seems to be pertinent to in the pathophysiology of various inflammatory diseases. Despite specific inhibitors were used to oppose the abnormal production of sPLA$_2$-IIA, it was ineffective to improve the clinical outcome for the patients with sever sepsis or rheumatoid arthritis [40, 43]. Therefore, improved approach needed for the cure of severe inflammatory diseases. Regarding this, FXa may be one of the candidates for the inhibition of sPLA$_2$-IIA expression. This concept is supported by the finding that sPLA$_2$-IIA transgenic mice develop hyper permeability [44] and sPLA$_2$-IIA itself directly induces the expression of chemokines and cell adhesion molecules in vascular endothelium [45]. In this perspective, FXa could be of special interest since in this study FXa showed the inhibitory effect of the expression and activity of sPLA$_2$-IIA.

In summary, the results presented in this study suggest that FXa can elicit inhibitory signaling responsive in sPLA$_2$-IIA expression and activity in cultured endothelial cells *via* interacting with its receptor, EPR-1, and mouse through the inhibition of ERK 1/2, cPLA2α, COX-2, and mPGES-1.

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Conflict of interest. The authors have no conflict of interest to declare.

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