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Inhibitory Effect of Thrombin on the Expression of 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) up-regulates the expression of sPLA$_2$-IIA in human umbilical vein endothelial cells (HUVECs). Recently, lower concentration thrombin could elicit anti-inflammatory responses in HUVECs. Here, the effects of lower concentration thrombin on the expression of sPLA$_2$-IIA in LPS-stimulated HUVECs were investigated. Prior treatment of cells with thrombin (25–75 pM) inhibited LPS-induced sPLA$_2$-IIA expression by activating its receptor, protease-activated receptor-1 (PAR-1). And pretreatment of cells with either PI3-kinase inhibitor (LY294002) or cholesterol depleting agent (methyl-β-cyclodextrin, MβCD) abolished the inhibitory activity of thrombin against sPLA$_2$-IIA expression. Therefore, these results suggest that PAR-1 activation by lower concentration thrombin inhibited LPS mediated expression of sPLA$_2$-IIA by PAR-1 and PI3-kinase-dependent manner in lipid raft on the HUVECs. J. Cell. Biochem. 112: 2502–2507, 2011.

KEY WORDS: THROMBIN; HUVEC; sPLA$_2$-IIA; PAR-1; LIPID RAFT

Phospholipases A_2 (PLA$_2$) makes up a superfamily of enzyme that hydrolyses the ester bond at the *sn*-2 position of phosphoglycerides to release free fatty acid and lysophospholipids [Six and Dennis, 2000; Kudo and Murakami, 2002]. This superfamily is divided into four groups by molecular weight and Ca^{2+}-dependence: secretory PLA$_2$ (sPLA$_2$), cytosolic PLA$_2$, Ca^{2+}-independent PLA$_2$, and lipoprotein-associated PLA$_2$ [Six and Dennis, 2000; Kudo and Murakami, 2002]. Although the biological functions of sPLA$_2$-IIA are not completely understood specifically, it might be involved in a variety of biological process in the mammalian cells such as coagulation, signal transduction, apoptosis, remodeling of cellular membranes, and host defense [Mounier et al., 1998; Murakami et al., 1998; Menschikowski et al., 2006]. And sPLA$_2$ is significantly associated with inflammatory diseases such as sepsis, bowel disease, acute pancreatitis, rheumatoid arthritis, bronchial asthma, and respiratory distress syndrome [Dennis, 1997]. In addition, large amounts of sPLA$_2$-IIA have been found in patients with severe inflammatory diseases including sepsis, septic shock, and ploytrauma suggesting important roles of sPLA$_2$-IIA in inflammation [Waydhas et al., 1989; Nakano et al., 1990; Crowl et al., 1991; Oka and Arita, 1991; Pruzanski and Vadas, 1991; Menschikowski et al., 2006].

Thrombin, a key enzyme of the blood coagulation cascade, was considered mainly as a pro-inflammatory factor [Coughlin, 2000] and specifically thrombin formed at a site of vascular damage activates numerous cells involved in the inflammatory responses [Cirino et al., 1996; Coughlin, 2001]. However, recently, anti-inflammatory activities of lower concentration thrombin such as blockade of leukocytes adhesion and migration to the damaged site, inhibition of cell adhesion molecules, and keeping barrier integrity in human endothelial cells were reported [Feistritzer and Riewald, 2005; Bae et al., 2007b, 2009]. So, there is now interest in studies on the role of thrombin in reactions limiting the inflammatory processes by inhibition of inflammatory mediators.

Since the induction of sPLA$_2$-IIA in endothelial cells is related with inflammation, in this study, it is hypothesized that lower concentration thrombin might reduce the expression levels of sPLA$_2$-IIA. Here, the expression of sPLA$_2$-IIA by lower concentration thrombin was monitored in endothelial cells in response to lipopolysaccharide (LPS). The results indicate that the cleavage of protease-activated receptor-1 (PAR-1) (thrombin receptor) by lower concentration thrombin inhibits sPLA$_2$-IIA expression levels in LPS-stimulated endothelial cells through phosphatidylinositol 3-kinase (PI3-kinase) and PAR-1 pathways and PAR-1 localization in lipid

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rafts is required for the inhibition signaling of $sPLA_2$-IIA expression. Based on these results, the cleavage of PAR-1 by lower concentration thrombin would initiate potent protective intracellular responses.

MATERIALS AND METHODS

REAGENTS

Thrombin, specific cell permeable phosphatidylinositol 3-kinase inhibitor (LY294002, used at 10 μM), cholesterol-depleting drug methyl-β-cyclodextrin (MβCD, used at 10 μM), and lipopolysaccharide (LPS, used at 50 ng/ml) were purchased from Sigma (St. Louis, MO). The cleavage blocking and nonblocking monoclonal anti-PAR-1 antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA) and used at 25 μg/ml each. PD98059 was purchased from Calbiochem (Schwalbach, Germany) and used at 25 μM. The thrombin receptor agonist peptide (TRAP) agonist peptide (TFLLRN) was purchased from Bachem Bioscience (Torrance, CA).

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 [Bae and Rezaie, 2009].

ELISA FOR $sPLA_2$-IIA

The level of $sPLA_2$-IIA protein in the cell culture medium and cell lysates was determined using specific ELISA kit (Cayman Chemical, Ann Arbor, MI) as described previously [Menschikowski et al., 2005; Bae and Rezaie, 2010] according to the manufacturer's instruction. Briefly, diluted medium or cell lysates containing 10–25 μg of total protein were 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. All values are expressed as pg $sPLA_2$ per mg total protein.

STATISTICAL ANALYSIS

Data are expressed as the means ± standard deviations 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

EFFECT OF VARYING THROMBIN CONCENTRATIONS ON THE $sPLA_2$-IIA ACTIVITY IN THE LPS-ACTIVATED HUVECs

It is well known that LPS and other inflammatory cytokines upregulate the transcription of sPLA-IIA and its protein level in a variety of cells including macrophage [Alaoui-El-Azher et al., 2002], fibroblast [Kuwata et al., 2007], endothelial cells [Flynn and Hoff, 1995], and astrocytes [Oka and Arita, 1991]. 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 both cell culture supernatants and cell lysates at 50 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 to serum of cell culture medium. Based on these results, LPS concentration at 50 ng/ml was used to stimulate endothelial for the further experiments described below.

It is well documented that thrombin mediates a pro-inflammatory response in HUVECs by up-regulating the activation of the NF-κB pathway by a PAR-1-dependent mechanism [Joyce et al., 1997; Lawrence et al., 2001; Macfarlane et al., 2001; Joyce and Grinnell, 2002]. Recently, thrombin (10 nM) did not have any effect on the expression of $sPLA_2$-IIA [Bae and Rezaie, 2010], however, lower concentration thrombin (50 pM) mediated anti-inflammatory responses in human endothelial cells [Feistritzer and Riewald, 2005; Bae et al., 2007b, 2009]. Thus, it is investigated whether lower concentration thrombin could modulate LPS-induced $sPLA_2$-IIA and found that thrombin at 25–75 pM potently inhibits the expression of $sPLA_2$-IIA in LPS-stimulated HUVECs (Fig. 1). Therefore, lower concentration thrombin inhibited both the released and cell associated forms of $sPLA_2$-IIA expression, suggesting a significance role of thrombin on the synthesis of this enzyme.

INHIBITORY EXPRESSION OF $sPLA_2$-IIA BY THROMBIN IS MODULATED BY A PAR-1 AND PI3-KINASE-DEPENDENT MANNER

It is known that thrombin elicits cellular responses in endothelial cells by activating PAR-1 and PI3-kinase [Ossovskaya et al., 2004; Mosnier et al., 2007; Bae et al., 2009; Bae and Rezaie, 2010]. Thus, it is determined whether the effect of thrombin in inhibition of $sPLA_2$-IIA in response to LPS is also mediated through the proteases activating PAR-1 and PI3-kinase. To do this, the same studies described above were conducted in the presence of function-blocking antibodies to both PAR-1 or specific cell permeable PI3-kinase inhibitor, LY294002. As shown in Figure 2, the down-regulated $sPLA_2$-IIA expression by thrombin was effectively suppressed by function-blocking antibodies to PAR-1 confirming that the cleavage of PAR-1 mediates the cellular effects of both proteases in the cell medium (Fig. 2A) or in the cell lysates (Fig. 2B) by PI3-kinase-dependent mechanism.

EFFECT OF MβCD ON THE MODULATION OF $sPLA_2$-IIA ACTIVITY BY THROMBIN

Recently, it was demonstrated that PAR-1 is associated with caveolin-1 within the lipid rafts of HUVECs and the anti-inflammatory effects of thrombin are abolished if endothelial cells are pretreated with cholesterol depleting agent, MβCD [Bae et al., 2007c; Bae and Rezaie, 2008]. To determine whether similar events are responsible for the inhibition of $sPLA_2$-IIA expression by lower concentration thrombin, endothelial cells were preincubated with MβCD. MβCD eliminated the inhibition activity of $sPLA_2$-IIA expression by thrombin in the cell medium (Fig. 2A) and in the cell lysates (Fig. 2B). These results suggest that cleavage of PAR-1 by

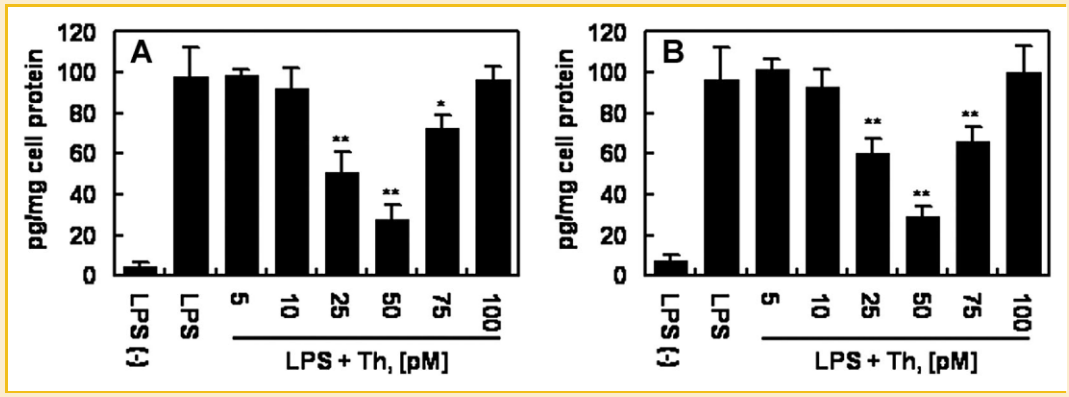

Fig. 1. Effect of thrombin on the expression of $sPLA_2$-IIA in endothelial cells. Primary HUVECs were preincubated with indicated concentrations of thrombin for 4 h. Then, cells were incubated with control serum-free media or 50 ng/ml LPS for 24 h followed by measuring the expression level of $sPLA_2$-IIA in culture medium (A) or in the cell lysates (B) as described under Materials and Methods Section. All results are shown as means ± SD of three different experiments. $^{**}P < 0.01$ as compared to LPS.

thrombin outside the lipid rafts initiates a pro-inflammatory response in endothelial cells.

DOWN-REGULATORY EFFECT OF THROMBIN COULD BE MIMICKED BY TRAP

It is well known that the TRAP TFLLRN specifically activates PAR-1 on endothelial cells [Ossovskaya et al., 2004; Steinhoff et al., 2005] and the activation of PAR-1 by the TRAP peptide is known to mimic the pro-inflammatory effect of thrombin in human endothelial cells [Shankar et al., 1994; Feistritzer and Riewald, 2005]. TRAP alone did not alter $sPLA_2$-IIA expression (data not shown). And lower concentration TRAP could inhibit LPS-induced $sPLA_2$-IIA expression in the cell medium (Fig. 3A) and in the cell lysates (Fig. 3B). And it is also tested the effect of functional-blocking antibodies against PAR-1, PI3-kinase inhibitor (LY294002), or MβCD on the inhibitory effect of TRAP on the LPS-induced $sPLA_2$-IIA expression. As expected, antibodies against PAR-1 were not effective because TRAP can activate PAR-1 regardless of cleavage of PAR-1 [Bae et al., 2007b; Bae and Rezaie, 2008]. And inhibitory effect of TRAP on the $sPLA_2$-IIA expression was also abolished by PI3-kinase inhibitor or MβCD in endothelial cells (Fig. 4).

The activation of PAR family by coagulation protease shows pivotal roles in mediating cellular responses in endothelial cells and other cells [Coughlin, 2005]. The common concept is that the cleavage of PAR-1 by either thrombin or TRAP elicits pro-inflammatory responses in vascular endothelial cells [Coughlin, 2005; Feistritzer and Riewald, 2005; Finigan et al., 2005; Komarova et al., 2007]. However, recent studies showed that lower concentration thrombin elicited anti-inflammatory responses [Feistritzer and Riewald, 2005; Bae et al., 2007b, 2009]. Here, the

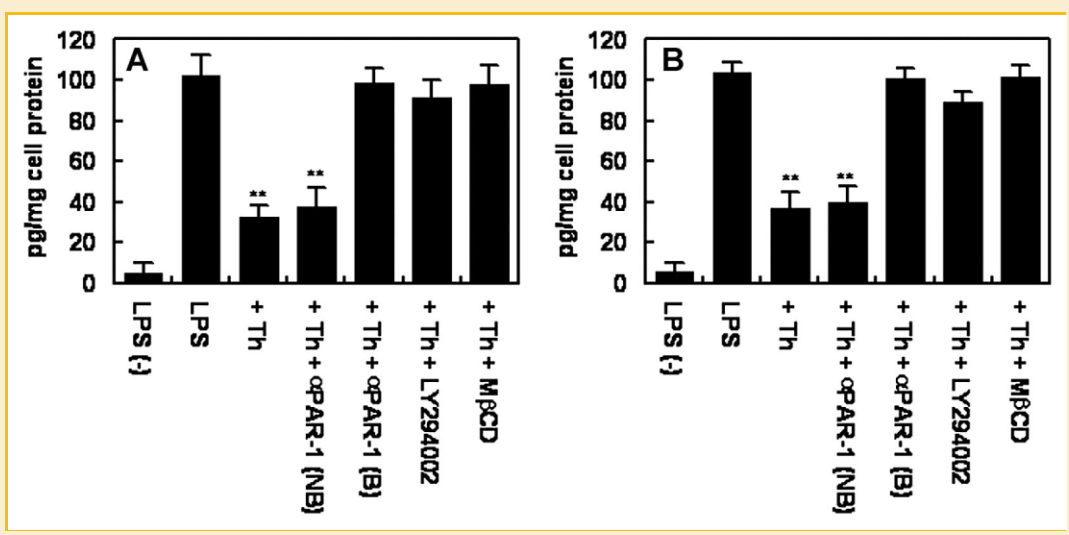

Fig. 2. The inhibitory effects of thrombin on the expression of $sPLA_2$-IIA are mediated through the PAR-1 and PI3-kinase. Primary HUVECs were preincubated with indicated functional nonblocking (NB) or blocking (B) antibodies for 30 min or with LY294002 (10 μM) for 1 h or with MβCD (10 mM) for 1.5 h. Then, cells were incubated with thrombin (50 pM) for 4 h. Cells were then incubated with control serum-free media, or 50 ng/ml LPS for 24 h followed by analysis of the expression level of $sPLA_2$-IIA in culture medium (A) or in the cell lysates (B). All results are shown as means ± SD of three different experiments. $^{**}P < 0.01$ as compared to LPS.

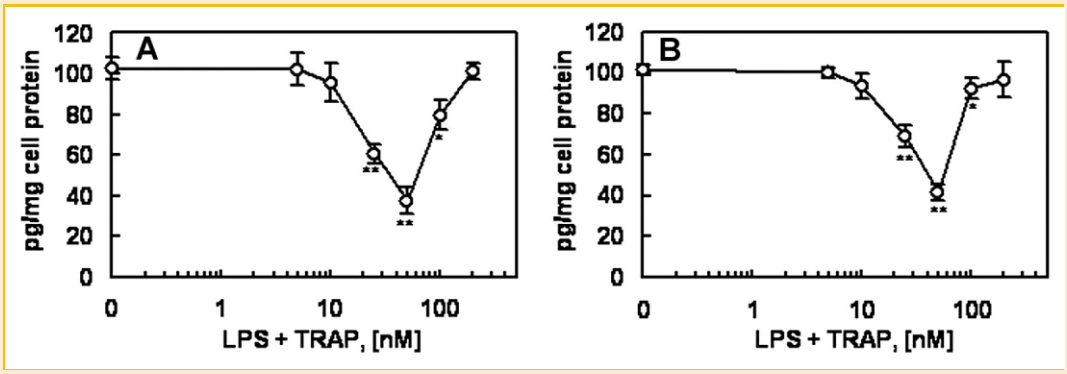

Fig. 3. Effect of TRAP on the expression of $sPLA_2$-IIA. Primary HUVECs were preincubated with indicated concentrations of TRAP for 4 h. Then, cells were incubated with control serum-free media or 50 ng/ml LPS for 24 h followed by measuring the expression level of $sPLA_2$-IIA in culture medium (A) or in the cell lysates (B) as described under Materials and Methods Section. All results are shown as means ± SD of three different experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to LPS (0 nM TRAP).

possible mechanisms according to which lower concentration thrombin down-regulates the $sPLA_2$-IIA expression were investigated. Previous studies demonstrated that the anti-inflammatory activities of thrombin are mediated through PAR-1-dependent manner [Feistritzer and Riewald, 2005; Bae et al., 2007a,b, 2009]. In the presence of functional-blocking antibodies against PAR-1, inhibition effects of $sPLA_2$-IIA expression by thrombin are markedly diminished suggesting that PAR-1 activation by thrombin mediates its effect on $sPLA_2$-IIA through PAR-1 pathways. Furthermore, the involvement of PI3-kinase in the anti-inflammatory activities of thrombin was suggested by previous study [Finigan et al., 2005; Bae et al., 2009]. In this study, PI3-kinase also involved in the inhibitory effect of $sPLA_2$-IIA expression by thrombin because when endothelial cells were pretreated with PI3-kinase inhibitors, LY294002, its effect on $sPLA_2$-IIA was markedly abolished. Further support for the suggestion that PAR-1 activation is involved in the thrombin mediated effects on $sPLA_2$-IIA in endothelial cells results from data showing that TRAP inhibits $sPLA_2$-IIA expression. The anti-inflammatory activity of lower concentration thrombin or TRAP requires the localization of both receptors to the lipid rafts of endothelial because the pretreatment of primary HUVECs with MβCD altered the inhibitory effect of $sPLA_2$-IIA expression.

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 [Waydhas et al., 1989; Nakano et al., 1990; Crowl et al., 1991; Oka and Arita, 1991; Pruzanski and Vadas, 1991; Menschikowski et al., 2006]. The expression level of $sPLA_2$-IIA is markedly induced by pro-inflammatory mediators and down-regulated by anti-inflammatory cytokines in a variety of cells and

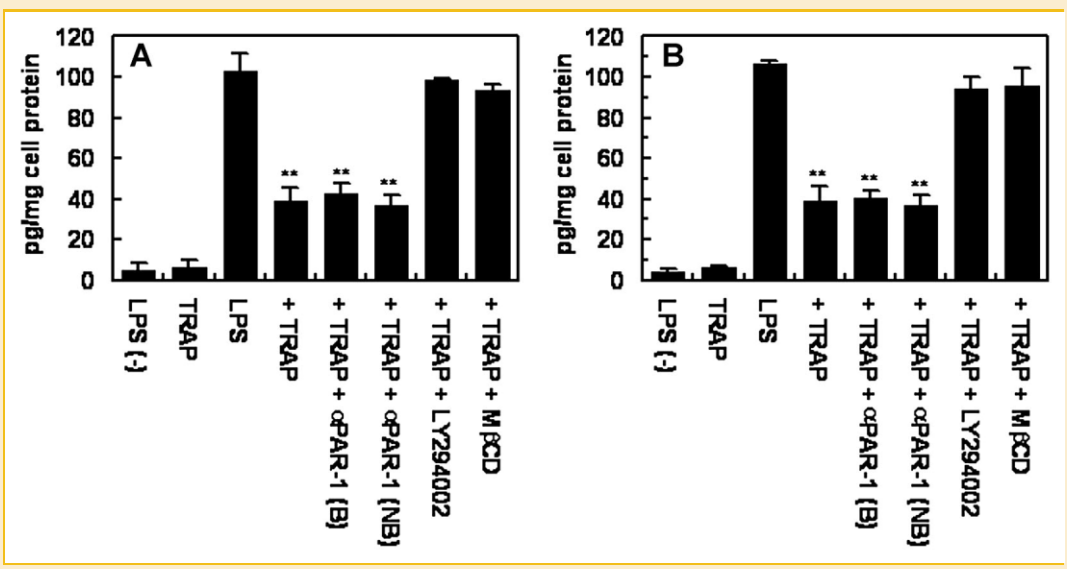

Fig. 4. The inhibitory effects of TRAP on the expression of $sPLA_2$-IIA are mediated through the PAR-1 and PI3-kinase. Primary HUVECs were preincubated with indicated functional nonblocking (NB) or blocking (B) antibodies for 30 min or with LY294002 for 1 h or with MβCD (10 mM) for 1.5 h. Then, cells were incubated with TRAP (50 nM) for 4 h. Cells were then incubated with control serum-free media, or 50 ng/ml LPS for 24 h followed by analysis of the expression level of $sPLA_2$-IIA in culture medium (A) or in the cell lysates (B). All results are shown as means ± SD of three different experiments. $^{**}P < 0.01$ as compared to LPS.

tissues in mammalians [Nakano et al., 1990; Crowl et al., 1991; Oka and Arita, 1991]. Therefore, the $sPLA_2$-IIA is thought to associate with the initiation and multiplication of inflammatory reactions. Supporting this, the inflammatory diseases are attenuated by $sPLA_2$-IIA inhibitors [Tanaka et al., 1993; Balsinde et al., 1999; Bradley et al., 2005], and in turn, purified $sPLA_2$-IIA aggravates these responses when injected into inflamed tissues [Vadas et al., 1989]. 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 of the patients with sever sepsis or rheumatoid arthritis [Bradley et al., 2005; Zeiher et al., 2005]. Therefore, improved approach needed for the cure of severe inflammatory diseases. Regarding this, pharmacological active agonists of PAR-1 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 [Grass et al., 1996] and $sPLA_2$-IIA itself directly induces the expression of chemokines and cell adhesion molecules in vascular endothelium [Beck et al., 2003]. In this perspective, lower concentration thrombin or TRAP could be of special interest because in this study lower concentration thrombin showed the inhibitory effect of $sPLA_2$-IIA expression.

In summary, the results presented in this study suggest that thrombin can elicit inhibitory signaling response in $sPLA_2$-IIA expression in cultured endothelial cells through the activation of PAR-1. It appears that the PAR-1-dependent signaling activity of thrombin in inhibitory signaling response in $sPLA_2$-IIA expression is mediated through a PI3-kinase-dependent activation and occurred only in the lipid rafts.

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