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Thrombin and activated protein C inhibit the expression of secretory group IIA phospholipase A₂ in the TNF- α -activated endothelial cells by EPCR and PAR-1 dependent mechanisms

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Abstract

Introduction—Thrombin and tumor necrosis factor (TNF)- α up-regulate the expression of proinflammatory molecules in human umbilical vein endothelial cells (HUVECs). However, activated protein C (APC) down-regulates the expression of the same molecules. The expression level of secretory group IIA phospholipase A₂ (sPLA₂-IIA) is known to be elevated in inflammatory disorders including in sepsis. Here, we investigated the effects of APC and thrombin on the expression of sPLA₂-IIA and extracellular signal-regulated kinase (ERK) in HUVECs.

Materials and methods—The expression level of sPLA₂-IIA was quantitatively measured by an enzyme-linked-immunosorbent-assay following stimulation of HUVECs with either thrombin or TNF- α in the absence and presence of the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY294002 and the cholesterol-depleting drug methyl- β -cyclodextrin (M β CD).

Results and conclusions—Thrombin had no effect on the expression of sPLA₂-IIA in HUVECs, however, TNF- α potently induced its expression. The prior treatment of cells with APC inhibited expression of sPLA₂-IIA through the EPCR-dependent cleavage of PAR-1. Further studies revealed that if HUVECs were pretreated with the zymogen protein C to occupy EPCR, thrombin also inhibited the TNF- α -mediated expression of sPLA₂-IIA through the cleavage of PAR-1. The EPCR-dependent cleavage of PAR-1 by both APC and thrombin increased the phosphorylation of ERK 1/2. Pretreatment of cells with either LY294002 or M β CD abolished the inhibitory activity of both APC and thrombin against sPLA₂-IIA expression, suggesting that the protein C occupancy of EPCR confers a PI3-kinase dependent protective activity for thrombin such that its cleavage of the lipid-raft localized PAR-1 inhibits the TNF- α -mediated expression of sPLA₂-IIA in HUVECs.

Keywords

Thrombin; APC; HUVEC; inflammation; PAR-1; PI3 kinase

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Introduction

Secretory phospholipase A₂ group IIA (sPLA₂-IIA) is a member of the secreted phospholipase family of lipid hydrolyzing enzymes that hydrolyses the ester bond at the *sn*-2 position of phosphoglycerides to release free fatty acid and lysophospholipids [1,2]. sPLA₂-IIA was initially isolated from the rheumatoid arthritic synovial fluid and platelets [3-5]. The biological function of sPLA₂-IIA is not well understood, though there are several reports hypothesizing a possible role for sPLA₂-IIA in the modulation of coagulation, signal transduction, apoptosis, remodeling of cellular membranes, and host defense systems [6-8]. A high expression level for this enzyme has been observed at inflamed sites and also in the serum of patients with inflammatory disorders such as sepsis, septic shock, polytrauma and atherosclerosis [9-12]. During acute or chronic inflammation, the level of sPLA₂-IIA expression has been shown to correlate with the severity of the disease [9-13]. Moreover, it has been demonstrated that sPLA₂-IIA promotes inflammation in animal models [14,15].

It is known that activated protein C (APC), in addition to its critical role in down-regulation of the clotting cascade, plays an important role in down-regulating the inflammatory pathways [16-18]. It is thought that the antiinflammatory activities of APC are responsible [16-18], at least partially, for its beneficial effect in reducing the mortality rate in patients with severe sepsis [19]. The antiinflammatory activities of APC are thought to be mediated through its interaction with endothelial protein C receptor (EPCR) and its subsequent cleavage of protease-activated receptor 1 (PAR-1) on endothelial cells [20]. It is known that the EPCR- and PAR-1-dependent protective signaling of APC results in the activation of the ERK 1/2 signaling pathway [21-23]. However, it is also known that PAR-1 activation by thrombin elicits potent proinflammatory responses in endothelial cells [24,25]. Recently, we showed that the occupancy of EPCR by the zymogen protein C (PC) changes the PAR-1-dependent signaling specificity of thrombin from a proinflammatory to an antiinflammatory response in cytokine stimulated endothelial cells [26-28]. We demonstrated that the occupancy of EPCR by protein C/APC regulates the interaction of EPCR with caveolin-1 within the membrane lipid-rafts, thereby regulating the signaling specificity of PAR-1 on endothelial cells independent of the protease (thrombin or APC) activating the receptor [28,29].

Noting the enhanced level of sPLA₂-IIA in inflammatory disorders and the ability of thrombin to initiate proinflammatory responses in cultured endothelial cells by activating the NF- κ B pathway [9-12,24,25,30-32], we initiated this study to determine whether thrombin can induce the expression of sPLA₂-IIA in endothelial cells by the same pathway, and if so, whether the APC-EPCR pathway can down-regulate this response. We discovered that TNF- α potently induced the expression of sPLA₂-IIA in endothelial cells, however, thrombin had no effect on its expression. APC effectively inhibited the TNF- α -mediated expression of sPLA₂-IIA in endothelial cells by a PAR-1-dependent manner. Interestingly, when EPCR was occupied by its ligand, the cleavage of PAR-1 by thrombin also inhibited the expression level of sPLA₂-IIA in TNF- α -stimulated endothelial cells. The same results were obtained if a thrombin receptor agonist peptide (TRAP) was used as the PAR-1 activator in endothelial cells. Further studies revealed that the EPCR- and PAR-1-dependent inhibitory effect of both APC and thrombin on endothelial cells is mediated through the activation of the phosphatidylinositol 3-kinase (PI3 kinase) and ERK 1/2 pathways. Based on these results, we conclude that the cleavage of PAR-1 by either APC or thrombin on endothelial cells expressing EPCR would reverse the proinflammatory effects of cytokines if EPCR was occupied by its natural ligand.

Materials and methods

Reagents

Tumor necrosis factor- α (TNF- α) was purchased from R&D System (Minneapolis, MN) and used at 10 ng/mL to 200 ng/mL. The cleavage blocking monoclonal anti-PAR-1 antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA) and used at 25 μ g/mL. The functional blocking anti-EPCR antibody (Clone RCR-252) was purchased from Cell Sciences (Canton, MA, USA) and used at 25 μ g/mL. Thrombin, specific cell permeable phosphatidylinositol 3-kinase inhibitor (LY294002, used at 10 μ M), cholesterol-depleting drug methyl- β -cyclodextrin (M β CD, used 10 mM) were obtained from Sigma (St. Louis, MO, USA). PD98059 was purchased from Calbiochem (Schwalbach, Germany) and used at 25 μ M. The thrombin receptor agonist peptide (TRAP) agonist peptide (TFLLRN, used at 100 μ M) was purchased from Bachem Bioscience (Torrance, CA). Expression and purification of wild type APC, zymogen protein C (PC) and the Ser-195 to Ala substitution mutant of PC (PC-S195A) have been described [28,29].

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science Inc. (Charles City, IA) and maintained as described as before [27-29]. Immortalized human umbilical vein endothelial cells (EA.hy926) was kindly provided by Dr. C. Edgell from University of North Carolina at Chapel Hill (NC, USA) and maintained as described before [26-29]. Briefly, Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin).

ELISA for sPLA₂-IIA and ERK 1/2 phosphorylation

The expression of sPLA₂-IIA protein was determined in the cell culture medium and cell lysates by using specific ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously [33] according to the manufacturer's instruction. The measurements were performed in duplicate in at least three independent experiments. Briefly, 50 μ L of diluted medium or cell lysates containing 5–25 μ g of total protein were added to each well of the plate coated with a monoclonal antibody specific for sPLA₂-IIA. Then, an acetylcholinesterase-sPLA₂-Fab' conjugate was added to each well after washing. The concentration of the analyte was then determined by measuring the enzymatic activity of the acetylcholinesterase 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. sPLA₂-IIA concentrations in the samples were calculated from a standard curve using recombinant sPLA₂-IIA as an a standard. The sensitivity of the assay was 5 pg/mL. To avoid possible non-specific reactions due to anti-mouse IgM or IgG heterophilic antibodies, each sample was treated with non-specific mouse serum prior to addition to the well. All values are expressed as pg sPLA₂ per mg total protein. Total cell protein concentration was determined by using Bradford protein assay with bovine serum albumin as an internal standard (Sigma, St. Louis, MO, USA).

For the measurement of ERK 1/2 phosphorylation, HUVECs were cultured in 96 well plates. On the day of the assay, the cell culture medium was replaced with fresh growth medium before treating the monolayer with appropriate reagents. PD98059 was added to HUVECs for 30 min prior to 5 min treatment with the proteases. The phosphorylation of ERK 1/2 was evaluated in the cell lysates by using specific Phospho ERK 1/2 ELISA kit (Thermo Scientific, Rockford, IL, USA) according to the manufacture's protocols.

Statistical Analysis

Results are expressed as means $\pm$ standard deviation (S.D.) of at least three independent experiments. The statistical significance of differences between test groups was used for statistical comparison (SPSS, version 14.0, SPSS Science, Chicago, IL, USA). Statistical relevance was determined using analysis of variance (ANOVA) with p-values less than 0.05 ($p < 0.05$).

Results and Discussion

TNF- α concentration-dependence of expression of sPLA₂-IIA by HUVECs

It is well known that TNF- α and other inflammatory cytokines up-regulate the transcription of sPLA₂-IIA and its protein level in a variety of cells including endothelial cells [34], vascular smooth muscle cells [35], chondrocytes [36], mesangial cells [37], astrocytes [38,39], and hepatoma cells [39]. The concentration-dependence of the TNF- α -mediated expression of sPLA₂-IIA in endothelial cells is presented in Fig. 1. Analysis of the expression level of sPLA₂-IIA by the transformed HUVECs in response to varying concentrations of TNF- α for 4 h indicated that the induction level reaches plateau in both cell culture supernatants (Fig. 1A) and cell lysates (Fig. 1B) at 100 ng/mL TNF- α . Similar expression levels in both supernatants and cell lysates suggest that the TNF- α -mediated enhancement of sPLA₂-IIA may not be due to its release from preformed storage vesicles, but rather due to the new synthesis of the molecule by the cytokine stimulated endothelial cells. A similar effect for TNF- α was observed if endothelial cells were cultured in serum free medium containing 0.2% BSA, excluding the possibility that the effect of TNF- α on sPLA₂-IIA expression is due to its interference with the function of some unknown factors in the serum. This effect was not specific for the transformed HUVECs since we essentially obtained identical results with primary HUVECs (data not shown). Based on these results, a TNF- α concentration of 100 ng/mL was used to stimulate endothelial cells in all experiments described below.

Effect of thrombin and APC on the sPLA₂-IIA activity

Previous results have indicated that thrombin initiates a proinflammatory response in HUVECs by up-regulating the activation of the NF- κ B pathway by a PAR-1-dependent mechanism [9-12,30-32]. Surprisingly, however, the incubation of HUVECs with thrombin (up to 10 nM) did not have any effect on the expression of sPLA₂-IIA (Fig. 2). Under these conditions, thrombin can activate all cell surface PAR-1 [23-25]. Thus, unlike the activation of the NF- κ B pathway, the activation of PAR-1 by thrombin does not lead to up-regulation of sPLA₂-IIA in HUVECs. A previous study showed that plasma derived APC effectively diminished the expression of sPLA₂-IIA by interferon- γ (INF- γ) in human aortic smooth muscle cells [40]. In this study, we investigated this question in HUVECs (both in transformed and primary cells) and found that APC potently inhibits the expression of sPLA₂-IIA in TNF- α -stimulated cells (Fig. 2). While thrombin had no effect on the expression of sPLA₂-IIA in response to TNF- α , it nevertheless, potently inhibited its expression if endothelial cells were pretreated with wild-type PC or PC-S195A prior to incubation of cells with thrombin and TNF- α (Fig. 2). A lack of activity for either PC or PC-S195A in this assay is not due to the weaker and/or the loss of the affinity of proteins for interaction with EPCR since both wild-type and mutant PC zymogens can bind to the receptor with a dissociation constant of ~ 30 nM that is essentially identical to the affinity of APC for the receptor [28,41,42]. Thus, in agreement with previous results, neither PC nor PC-S195A can elicit PAR-1-dependent cellular response in HUVECs [28,41,42].

The expression of sPLA₂-IIA is modulated by an EPCR- and PAR-1-dependent mechanism

It is known that both APC and thrombin elicit cellular responses in endothelial cells by activating PAR-1 [17,43]. To determine whether the effect of APC and thrombin + PC-S195A in inhibition of sPLA₂-IIA in response to TNF- α is also mediated through the proteases activating PAR-1, the same studies described above were conducted in the presence of function-blocking antibodies to both PAR-1 and EPCR. As shown in Fig. 3, function-blocking antibodies to both EPCR and PAR-1 eliminated the inhibitory effects of both APC and thrombin on the expression of sPLA₂-IIA, suggesting that the EPCR-dependent cleavage of PAR-1 mediates the cellular effects of both proteases (Fig. 3).

PI3 kinase modulates the expression of sPLA₂-IIA

Previous studies have indicated that the PAR-1-dependent cellular effect of both thrombin and APC is mediated through the activations of the PI3 kinase [25,44] and ERK 1/2 pathways [21-23]. However, zymogen PC could not alter PI3 kinase pathway [25]. To determine whether PI3 kinase is also involved in the inhibition of sPLA₂-IIA expression by APC and thrombin + PC-S195A, endothelial cells were preincubated with specific PI3-kinase inhibitor, LY294002. As presented in Fig. 4, the inhibitor effectively suppressed the sPLA₂-IIA modulatory effects of both APC and thrombin in TNF- α -stimulated endothelial cells, suggesting that this effect is mediated through the proteases activating the PI3 kinase pathway by an EPCR- and PAR-1-dependent mechanism. The activation of the PI3 kinase pathway by APC requires an intact active site since the zymogen PC cannot alter this pathway [25].

To determine whether, similar to APC, the phosphorylation of ERK 1/2 is involved in the EPCR-dependent protective signaling effect of thrombin, we quantitatively analyzed the extent of phosphorylation of ERK 1/2 by using Phospho ERK 1/2 ELISA kit. As shown in Fig. 5, both APC and thrombin + PC-S195A, but not PC or PC-S195A alone, up-regulated the intracellular levels of phosphorylated ERK 1/2 in transformed HUVECs. Consistent with these results, the ERK 1/2 inhibitor (PD98059) prevented the APC or thrombin + PC-S195A induced ERK 1/2 phosphorylation in HUVECs (Fig. 5). Based on these results, we conclude that the inhibitory effect of both APC and thrombin + PC-S195A on the expression of sPLA₂-IIA are mediated through the activation of the ERK 1/2 pathway. As with all other experiments in this manuscript, we confirmed these results are all applicable to primary HUVECs (data not shown).

Effect of M β CD on the modulation of sPLA₂-IIA activity by APC and thrombin

Recently, we demonstrated that EPCR and PAR-1 are associated with caveolin-1 within the lipid rafts of HUVECs and that the antiinflammatory effects of APC or thrombin + PC-S195A are abolished if endothelial cells are pretreated with cholesterol depleting agent, methyl- β -cyclodextrin (M β CD) [26,29]. To determine whether similar events are responsible for the EPCR- and PAR-1-dependent inhibition of sPLA₂-IIA expression by APC or thrombin, endothelial cells were preincubated with M β CD. As shown in Fig. 4, M β CD eliminated the inhibitory activity of both APC and thrombin on the expression of sPLA₂-IIA, suggesting that the cleavage of PAR-1 by either protease outside the lipid rafts has no protective effect against the proinflammatory effect of TNF- α in endothelial cells. However, when EPCR is occupied, the activation of the lipid-raft localized PAR-1 by either thrombin or APC elicits protective responses, thereby inhibiting the expression of proinflammatory molecules by the cytokine-activated endothelial cells. In a previous study we showed that both unoccupied EPCR and PAR-1 are associated with caveolin-1 within lipid rafts of endothelial cells [26,28]. However, when EPCR is occupied by protein C, the receptor dissociates from caveolin-1, and this process appears to be linked to changing the G-protein coupling specificity of PAR-1 so that its cleavage by either thrombin or APC leads to initiation of protective intracellular responses in endothelial cells [26,28]. We believe that a similar mechanism accounts for the EPCR- and PAR-1-

dependent thrombin suppression of TNF- α -mediated up-regulation of sPLA₂-IIA in endothelial cells.

The modulatory effect of proteases on the expression of sPLA₂-IIA can be mimicked by TRAP

It is well known that the thrombin receptor agonist peptide (TRAP) TFLLRN specifically activates PAR-1 on endothelial cells [43,45] and the activation of PAR-1 by the TRAP peptide is known to mimic the proinflammatory effect of thrombin in HUVECs [46,47]. As presented in Fig. 6, TRAP alone could not inhibit the TNF- α induced sPLA₂-IIA expression. However, as observed with thrombin, if endothelial cells were treated with PC-S195A, TRAP inhibited the TNF- α -induced sPLA₂-IIA expression, supporting the conclusion that when EPCR is occupied by its ligand, PAR-1 activation reverses the proinflammatory response of TNF- α in endothelial cells (Fig. 6). TRAP alone had no effect on the expression of sPLA₂-IIA (Fig. 6). We also tested the effect of function-blocking antibodies against both PAR-1 and EPCR. As expected, the antibody against PAR-1 was not effective because TRAP can activate PAR-1 independent of its cleavage [26,28]. However, the inhibitory effect of TRAP with PC-S195A on the expression of sPLA₂-IIA was abolished by the anti-EPCR antibody, supporting the hypothesis that the occupancy of EPCR modulates the signaling specificity of PAR-1. Consistent with the involvement of the PI3 kinase pathway in the PAR-1-dependent protective response, the PI3 kinase inhibitor, LY294002, inhibited the activity of TRAP in endothelial cells (Fig. 6). These results were reproducible in primary HUVECs (data not shown).

Noting that neither thrombin nor TRAP had any effect on the TNF- α -mediated expression of sPLA₂-IIA, the results presented above suggest that the activation of PAR-1 on endothelial cells has no modulatory effect on the expression of this molecule unless EPCR is occupied by its physiological ligand. We previously demonstrated that the occupancy of EPCR by protein C switches the PAR-1-dependent signaling specificity of thrombin from a proinflammatory response to an inflammatory response in cultured endothelial cells [26-28]. Thus, consistent with the literature [26-28], in our previous studies we showed that thrombin activates the NF- κ B pathway and elevates the expression level of related proinflammatory molecules including the expression of cell adhesion molecules in endothelial cells. PC nor PC-S195A alone could not alter NF- κ B pathway [27]. However, we showed that when endothelial cells are pretreated with a catalytically inactive S195A mutant of the zymogen protein C so that to occupy EPCR, the activation of PAR-1 by thrombin can no longer activate the NF- κ B pathway, but rather it elicits a protective response thus inhibiting the activation of the proinflammatory pathways in the cytokine stimulated endothelial cells [27]. It is already known that the TNF- α mediated activation of the NF- κ B pathway is required for the induction of sPLA₂-IIA gene expression [48]. Thus, based on our results here and those published previously [48], we hypothesize that the EPCR and PAR-1-dependent activations of PI3 kinase and ERK 1/2 pathways inhibit the expression of sPLA₂-IIA through down-regulation of the TNF- α -mediated NF- κ B pathway.

sPLA₂-IIA has been hypothesized to play a role in regulating inflammation. Thus, a high level of this molecule has been found in the sera of patients with inflammatory disorders with its level apparently correlating with the severity of inflammation [9-12]. However, the possibility that sPLA₂-IIA is only an inflammatory marker rather than a contributor to inflammation, has not been ruled out. Given that the activation of PAR-1 can activate the NF- κ B pathway [9-12,30-32], the observations that neither thrombin nor TRAP induced the expression level of sPLA₂-IIA in endothelial cells suggest that, unlike other proinflammatory molecules, the sPLA₂-IIA gene may not be modulated through the NF- κ B pathway. Thus, further studies will be required to understand how TNF- α up-regulates the expression of sPLA₂-IIA in endothelial cells.

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Abbreviations

sPLA ₂ -IIA	secretory phospholipase A ₂ of group IIA
TNF- α	tumor necrosis factor alpha
PC	protein C
APC	activated protein C
PAR-1	protease-activated receptor-1
ELISA	enzyme-linked immunosorbent assay
EPCR	endothelial protein C receptor
HUVEC	human umbilical vein endothelial cell
TRAP	thrombin receptor agonist peptide
BSA	bovine serum albumin
Th	thrombin
PI3	phosphatidylinositol 3
PC-S195A	Ser-195 to Ala substitution mutant of protein C
ERK 1/2	extracellular signal-regulated kinase

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Figure 1. Inducible effect of TNF- α on the expression of sPLA₂-IIA in endothelial cells

Transformed HUVECs were incubated with indicated concentrations of TNF- α for 4 h followed by measuring the expression level of sPLA₂-IIA in the cell culture medium (A) or in the cell lysates (B) as described under "Materials and Methods". All results are shown as means $\pm$ SD of three different experiments. * $p < 0.05$ as compared to 0 ng/mL TNF- α .

Figure 2. Effect of APC or thrombin on the expression of sPLA₂-IIA in HUVECs pretreated with PC-S195A

Transformed HUVECs were preincubated with PC (80 nM), PC-S195A (80 nM), APC (20 nM), Th (2 nM), and Th plus PC-S195A for 6 h. Then, cells were incubated with control serum free media or 100 ng/mL TNF- α for 4 h followed by measuring the expression level of sPLA₂-IIA in culture medium (A) or in the cell lysates (B) as described under “Materials and Methods”. All results are shown as means $\pm$ SD of three different experiments. * $p < 0.05$ as compared to 100 ng/mL TNF- α .

Figure 3. The inhibitory effects of both APC and thrombin on the expression of sPLA₂-IIA are mediated through the PAR-1 and EPCR pathways

Transformed HUVECs were preincubated with indicated function-blocking antibodies for 30 min. Then, cells were incubated with APC (20 nM) or Th (2 nM) plus PC-S195A (80 nM) for 6 h. Cells were then incubated with control serum free media, or 100 ng/mL TNF- α for 4 h followed by analysis of the expression level of sPLA₂-IIA in culture medium (A) or in the cell lysates (B). All results are shown as means $\pm$ SD of three different experiments. * $p < 0.05$ as compared to 100 ng/mL TNF- α .

Figure 4. Effect of the PI3 kinase inhibitor (LY294002) and methyl- β -cyclodextrin (M β CD) on the expression of sPLA₂-IIA in endothelial cells

Transformed HUVECs were preincubated with LY294002 (10 μ M) for 1 h or M β CD (10 mM) for 1.5 h followed by monitoring the expression levels of sPLA₂-IIA by 100 ng/mL TNF- α for 4 h in the presence of APC (20 nM) or Th (2 nM) plus PC-S195A (80 nM) in culture medium (A) or in the cell lysates (B). All results are shown as means $\pm$ SD of three different experiments.

* $p < 0.05$ as compared to 100 ng/mL TNF- α .

Figure 5. Effects of APC and thrombin + PC-S195A on the phosphorylation of ERK 1/2
Transformed HUVECs were preincubated with indicated proteases for 5 min and 25 μ M PD98059 (ERK 1/2 inhibitor) was added to HUVECs prior to treatment of the monolayer by the proteases. Phosphorylated ERK 1/2 was measured by an ELISA assay as described under “Materials and Methods”. All results are shown as means $\pm$ SD of three different experiments.
* $p < 0.05$ as compared to control.

Figure 6. Effect of TRAP on the expression of sPLA₂-IIA

Transformed HUVECs were preincubated with indicated function-blocking antibodies for 30 min or LY294002 for 1 h. Then, cells were incubated with TRAP (100 μ M) alone or together PC-S195A for 6 h. Cells were then incubated with control serum free media, or 100 ng/mL TNF- α for 4 h followed by analysis of the expression level of sPLA₂-IIA in culture medium (A) or in the cell lysates (B). All results are shown as means $\pm$ SD of three different experiments.

* $p < 0.05$ as compared to 100 ng/mL TNF- α .