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Inhibitory effects of antithrombin on the expression of secretory group IIA phospholipase A_2 in endothelial cells

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Tumor necrosis factor-α (TNF-α) mediates proinflammatory responses in primary human umbilical vein endothelial cells (HUVECs), and it upregulates the expression of secretory group IIA phospholipase A_2 ($sPLA_2$-IIA). $sPLA_2$-IIA plays a pivotal role in inflammation, and antithrombin (AT) possesses properties that are beneficial to endothelial cells. Therefore, we investigated the effects of AT on the expression of $sPLA_2$-IIA in TNF-α-stimulated HUVECs. TNF-α potently upregulated the expression of $sPLA_2$-IIA, and prior treatment of cells with AT inhibited the expression of $sPLA_2$-IIA in HUVECs. Also, antibodies or siRNA for syndecan-4 blocked the protective effect of AT. Furthermore, PI3-kinase and the AKT pathway are significantly involved in the AT-mediated inhibition of the expression of $sPLA_2$-IIA. These results show that AT effectively suppresses the upregulated $sPLA_2$-IIA expression, which might contribute to the cytoprotective effects of AT in the treatment of severe inflammatory diseases. [BMB reports 2010; 43(9): 604-608]

INTRODUCTION

Phospholipases A_2 (PLA_2) comprise a superfamily of enzymes that hydrolyze the ester bond at the *sn*-2 position of phosphoglycerides to release free fatty acid and lysophospholipids (1). Secretory PLA_2 ($sPLA_2$) is a small, 14 kDa enzyme found in the venom of certain snakes (types IA, IIA, and IIB); pancreatic juices (type IB); rat and mouse testes (type IIC); placenta tissue; synovial fluids and platelets (type IIA); and heart, lung, and P388D1 macrophages (type V) (1). These isoenzymes are associated with various activities such as the production of lipid mediators contributing to inflammation, tumorigenesis, bacterial defense, fertilization, and phospholipid digestion in the gastrointestinal track (1). Although the biological functions of $sPLA_2$-IIA are not completely elucidated, 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 (2). In addition, large amounts of $sPLA_2$-IIA have been found in patients with severe inflammatory diseases (e.g. sepsis, septic shock, ploytrauma), suggesting $sPLA_2$-IIA involvement in inflammation (2-4).

AT is a physiological anticoagulant present in human plasma, and it regulates the proteolytic activity of serine proteases in both intrinsic and extrinsic pathways (5). As part of its anticoagulating activity, AT binds to the heparin-like glycosaminoglycans (GAGs) in the microvasculature (5). In addition to its anticoagulant activity, AT also has antiinflammatory properties (6). In the sepsis model, lipopolysaccharide (LPS) mediates sepsis syndrome by activating monocytes to produce proinflammatory cytokines such as TNF-α (7). TNF-α plays pivotal roles in the development of septic shock and multi organ failure by activating neutrophils and by upregulating nitric oxide synthase (7).

Syndecan-4 is a transmembrane heparan sulfate proteoglycan belonging to the syndecan family (8). Heparan sulfate chains of syndecan-4 are believed to play numerous roles by binding to growth factors such as basic fibroblast growth factor (bFGF) and midkine, anticoagulation factors such as tissue factor pathway inhibitor, and cell adhesion molecules such as fibronectin (9). It has been proposed that syndecan-4 serves as a signaling cell surface receptor for AT on endothelial cells and leukocytes, thus altering migratory responses and cell-cell adhesion (6, 10).

Recently, we demonstrated that AT reduces LPS-induced inflammatory responses such as barrier protective effect, upregulation of prostacyclin, inhibition of neutrophil adhesion to endothelial cell, inhibition of the expression of extracellular matrix protein, and down-regulation of nuclear factor kappa B (NF-κB) through its receptor, syndecan-4, in human endothelial cells (ECs) (11). However, it has not been reported whether or not AT exerts direct or indirect inhibitory effects on $sPLA_2$-IIA production in human endothelial cells through its receptor, syndecan-4. In the present study, we investigated the effect of AT on $sPLA_2$-IIA production by TNF-α-activated human endothelial cells in the absence or presence of siRNA against syndecan-4 or the presence of antibodies toward syndecan-4. In addition, we determined the mechanism by which AT mediated

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inhibitory effects on the expression of sPLA$_2$-IIA in TNF-α-stimulated HUVECs.

RESULTS AND DISCUSSION

It is well known that TNF-α and other inflammatory cytokines mediate the expression of sPLA$_2$-IIA protein levels in a variety of cells, including endothelial cells (3), vascular smooth muscle cells (12), and astrocytes (3, 4). To achieve elevated levels of sPLA$_2$-IIA (as occurs during inflammation), HUVECs were stimulated with TNF-α. Recently, we showed that TNF-α upregulated the expression levels of sPLA$_2$-IIA in primary HUVECs (13). To confirm this response in Ea.hy926 cells (transformed HUVECs), cells were activated with TNF-α in a concentration-dependent manner, as shown in Fig. 1A and B. Determination of the Ea.hy926 cells' expression of sPLA$_2$-IIA in response to different concentrations of TNF-α for four hours indicated that the induction level reaches a plateau in both cell culture supernatants (Fig. 1A) and cell lysates (Fig. 1B) at 100 ng/ml TNF-α Based on these results, a TNF-α concentration of 100 ng/ml was used to stimulate endothelial cells in all experiments described below. These culture conditions were consistent with those used in a previous study, in which primary HUVECs were treated with TNF-α 100 ng/ml for four hours (14). To clarify whether or not the effect of TNF-α on sPLA$_2$-IIA expression was due to the new synthesis of the molecule, Ea.hy926 cells were treated with a protein synthesis inhibitor, cycloheximide. As shown in Fig. 1C and D, cycloheximide inhibited the expression level of sPLA$_2$-IIA by TNF-α in both supernatants and cell lysates, suggesting that TNF-α mediated upregulation of sPLA$_2$-IIA was due to the new synthesis and secretion of the sPLA$_2$-IIA.

A recent study showed that plasma-derived activated protein C (APC) effectively down-regulated the expression of sPLA$_2$-IIA by interferon-γ (INF-γ) in human aortic smooth muscle cells (12). However, the effects of AT on sPLA$_2$-IIA in cytokine-stimulated HUVECs have not been studied. In this study we investigated this phenomenon in HUVECs (in both transformed and primary cells) and found that AT efficiently inhibits the expression of sPLA$_2$-IIA in TNF-α-stimulated Ea.hy926 cells (Fig. 2A, B) and primary HUVECs (Fig. 2C, D).

Next, we determined the IC$_{50}$ of AT on the TNF-α-induced sPLA$_2$-IIA stimulation (Table 1). The IC$_{50}$ of AT is lower than the physiological concentration of antithrombin (150 μg/ml, 1 U/ml) in both Ea.hy926 cells and primary HUVECs. These results suggest that the inhibitory effect of AT on sPLA$_2$-IIA is physiologically relevant.

Noting that syndecan-4 mediates the cellular effects of AT (6), we investigated the effect of siRNA for syndecan-4 on AT-mediated inhibition of sPLA$_2$-IIA. The transfection efficacy of siRNA for syndecan-4 and the knockdown expression of syndecan-4 in primary HUVECs were determined (Fig. 3A). As shown in Fig. 3B, the inhibitory effect of AT on TNF-α-activated, primary HUVECs was significantly diminished when HUVECs were pretreated with the siRNA to syndecan-4, sug-

Table 1. Inhibitory effect of AT on TNF-a induced sPLA$_2$-IIA

AT on human EC		IC50 (U/ml)*
Primary HUVEC	Supernatant	0.32 †
	Lysate	0.29
Ea.hy926	Supernatant	0.27
	Lysate	0.24

*Each value represents the mean ± SD (n=3), †Each value expressed in units of AT.

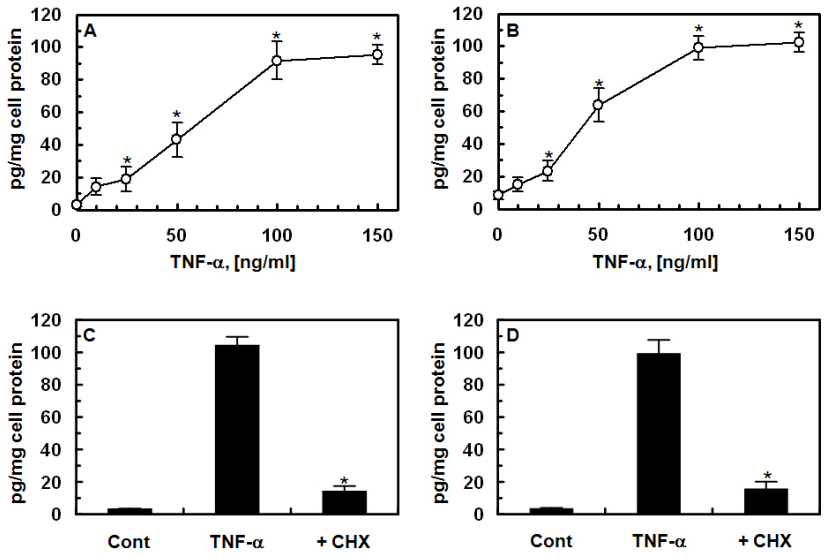

Fig. 1. Effect of TNF-α on the expression of sPLA$_2$-IIA. Ea.hy926 cells were incubated with the indicated concentrations of TNF-α for four hours, followed by measurement of the expression level of sPLA$_2$-IIA in the cell culture medium (A) or in the cell lysates (B), as described in "Materials and Methods". *P < 0.05 as compared to 0 ng/ml TNF-α. Preincubation of Ea.hy926 cells with cycloheximide (CHX, 5 μg/ml) was performed for four hours. After washing with PBS, cells were incubated with TNF-α 100 ng/ml for an additional four hours, followed by measurement of sPLA$_2$-IIA in the cell culture medium (C) or in the cell lysates (D), as described above. All results are shown as means ± SD of three different experiments. *P < 0.05 as compared to 0 ng/ml TNF-α.

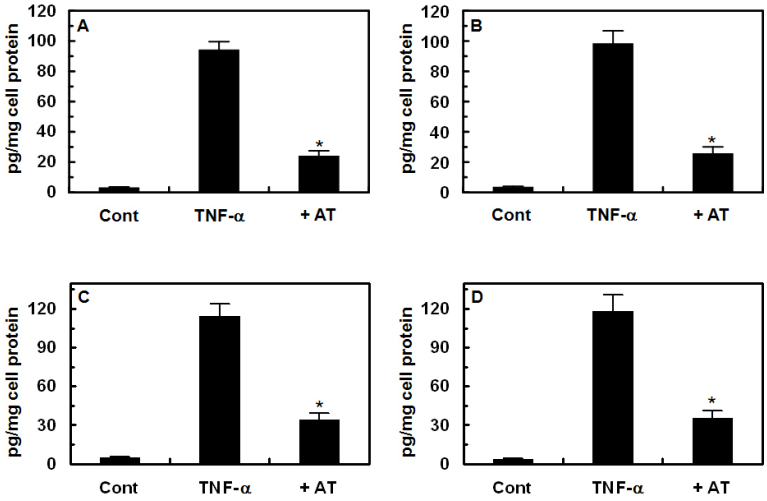

Fig. 2. Effect of AT on the expression of $sPLA_2$-IIA. Ea.hy926 cells (A, B) or primary HUVECs (C, D) were preincubated with AT (150 μg/ml) for six hours. Next, cells were incubated with serum-free media (control) or 100 ng/ml TNF-α for four hours, followed by measurement of the expression level of $sPLA_2$-IIA in culture medium (A, C) or in the cell lysates (B, D), as described in "Materials and Methods". All results are shown as means ± SD of three different experiments. *$P < 0.05$ as compared to 100 ng/ml TNF-α.

gesting that interaction with this receptor is required for the antiinflammatory effect of AT. To confirm the involvement of syndecab-4 in the AT-mediated inhibitory effect on $sPLA_2$-IIA, $sPLA_2$-IIA expression experiments were performed in the presence of AT using the antibody to syndecan-4 (D-16). A previous study confirmed the functional blocking of syndecan-4 by D-16 in endothelial cells (10). Primary HUVECs were pretreated with the antibody (D-16) or the isotype-matched IgG and were activated with TNF-α. Treatment of primary HUVECs with the antibody to syndecan-4 (but not the control IgG) significantly abolished the AT-mediated down-regulation of $sPLA_2$-IIA (Fig. 3B). Therefore, the finding that siRNA or the antibody for syndecan-4 blocked the inhibitory activity of AT suggests that AT interaction with syndycan-4 is essential for the antiinflammatory effect of AT.

We investigated how antithrombin suppresses TNF-α-induced $sPLA_2$-IIA. Cross talk between the MEK 1/2 and PI3- kinase/Akt pathways has been reported: inhibition of the PI3K/Akt pathway results in acceleration of MEK/ERK signaling and concomitant augmentation of proinflammatory cytokines in human epithelial cells, and the MEK 1/2 pathway negatively regulates PI3-kinase/Akt signaling in NIH313 cells (15). Furthermore, PI3-kinase, AKT, and MAP kinase have been implicated in the regulation of survival in several cell types (16-18). We have shown previously that PI3-kinase was involved in the APC or in lower concentration, thrombin-mediated, anti-inflammatory activities (13, 19). However, it is still unknown whether PI3-kinase, AKT, and MAP kinase are required for AT-mediated antiinflammatory responses in TNF-α-induced HUVECs. To solve this question, we investigated whether PI3-kinase, AKT, and MAP kinase have a role in AT-mediated inhibitory effects on TNF-α-induced $sPLA_2$-IIA expression via specific inhibitors in primary HUVECs (Fig. 3C). The PI3-kinase and AKT inhibitors abolished the AT-mediated inhibitory effect; however, the MEK1/2 inhibitor had no effect in these experiments (Fig. 3C). Taken together, our findings suggest that the PI3-kinase/AKT pathway promotes AT mediated inhibitory effects on TNF-α-induced $sPLA_2$-IIA expression but not MEK1/2 dependent expression.

$sPLA_2$-IIA seems to play a role in the initiation and propagation of inflammation (12), and a high level of this molecule has been found in the sera of patients with inflammatory disorders (2-4, 20, 21). However, the possibility that $sPLA_2$-IIA is only an inflammatory marker - rather than a contributor to inflammation - has not been ruled out because selective $sPLA_2$-IIA inhibitors were used to treat septic or rheumatoid arthritis patients to oppose the abnormal production of $sPLA_2$-IIA but failed to improve the clinical outcome (22, 23).

Antiinflammatory effects of AT are primarily attributed to a promotion of endothelial release of prostacyclin, and a prostacyclin application mimicked some of the effects of AT (11, 24). In addition, similar results were drawn by Hoffmann et al. (2002), who observed prostacyclin-mediated reduction of leukocyte adhesion to endothelial cells (25). It was also observed that AT inhibited the transendothelial migration towards endothelia in a syndecan-4 dependent manner (6, 26). In the present study we demonstrated that AT is capable of inhibiting $sPLA_2$-IIA production in TNF-α-activated HUVECs via interaction with its receptor syndecan-4 via PI3-kinase/AKT pathway. Therefore, the inhibitory effect of AT on the expression of $sPLA_2$-IIA may contribute to the antiinflammatory effects of AT in the endothelium.

MATERIALS AND METHODS

Reagents

TNF-α was obtained from R&D System (Minneapolis, MN, USA) and used at 10 ng/ml to 150 ng/ml. Human antithrombin III (AT) was purchased from Haematologic Technologies, Inc. (Essex Junction, VT, USA) and used at 150 μg/ml (1 U/ml) for

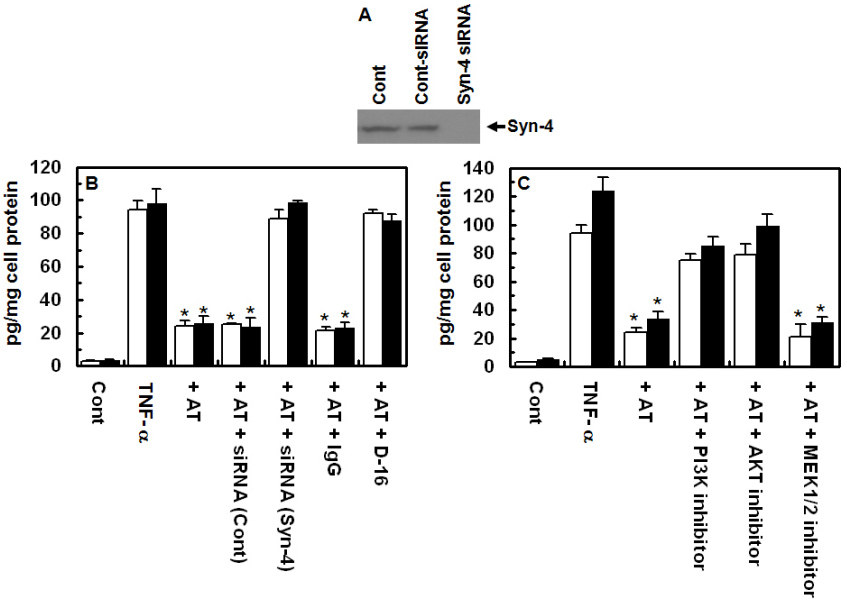

Fig. 3. Involvement of syndecan-4 in AT activity and the effects of signal pathway inhibitors on AT activity. (A) A 10% SDS-PAGE and western-blot of siRNA treated cells developed with antibodies directed to syndecan-4. (B) Primary HUVECs were preincubated with AT (150 μg/ml) for six hours in the absence or presence of siRNA for NS or syndecan-4 (Syn-4) or control (IgG or antibody for syndecan-4 (D-16)). Next, cells were incubated with serum-free media (control) or 100 ng/ml TNF-α for four hours, followed by measurement of the expression level of $sPLA_2$-IIA in culture medium (□) or in the cell lysates (■), as described in "Materials and Methods". (C) The same as (B) except the cells were preincubated with AT in the absence or presence of inhibitors for PI3-kinase, AKT, and MEK1/2. All results are shown as means ± SD of three different experiments. *P < 0.05 as compared to 100 ng/ml TNF-α.

four hours. siRNA for non-specific (NS) binding and syndecan-4 were obtained from Santa Cruz Biologics (Santa Cruz, CA, USA). Anti-syndecan-4 antibody (D-16) was purchased from Santa Cruz Biologics (Santa Cruz, CA, USA) and used at 2 μg/ml. Cycloheximide (used at 5 μg/ml), LY-294002 (PI3 kinase inhibitor, used at 10 μM), and U0126 (MEK1/2 inhibitor, used at 10 μM) were obtained from Sigma (St. Louis, MO, USA). 124005 (AKT inhibitor, used at 10 μM) was purchased from Calbiochem (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 (19). Immortalized human umbilical vein endothelial cells (EA.hy926) were kindly provided by Dr. C. Edgell from the University of North Carolina at Chapel Hill (NC, USA) and maintained as described previously (19). Briefly, cells were cultured at 37°C in Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) and supplemented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin).

Inhibition assay (IC_{50})

The 50% inhibiting concentration (IC_{50}) values of the high inhibition ratio were determined by monitoring the inhibitory effect of AT on TNF-α-induced $sPLA_2$-IIA protein levels with increasing concentrations and calculated using the Grafit program 3.01 (Erithacus Software Corp., Horley, Surrey, UK).

Western blotting for Syn-4

Equal amounts of total cellular protein were subjected to 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Bio-Rad). Blots were blocked overnight at 4°C with 5% skim milk and were incubated with primary antibodies for Syn-4. Blots were incubated further with diluted (1 : 2,000) horseradish peroxidase-conjugated secondary antibodies (Sigma, St Louis, MO). Immunoreactive protein bands were visualized using SuperSignal West Pico (Pierce, Pockford, IL). Signal intensities were quantified by densitometry (Gel-Pro Analyzer).

siRNA transfection

The effect of AT in response to TNF-α was evaluated following the knockdown of syndecan-4 expression by target-specific, 20-25 necleotide siRNA (used 0.2 μg/ml, for three hours, Santa Cruz Biologics, Santa Cruz, CA, USA) according to the manufacturer's instructions, as described previously (11). A non-targeting, 20-25 nucleotide siRNA obtained from the same company was used as a negative control.

ELISA for $sPLA_2$-IIA

The amount of $sPLA_2$-IIA protein was determined in the cell culture medium and cell lysates using a specific ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), as described previously (13), according to the manufacturer's instruction. The total volume of cell lysates or media assayed was 200 μl. Total cell protein was determined using a Bradford protein assay with bovine serum albumin as an internal standard (Sigma, St. Louis, MO, USA). The calculated results were shown as pg of $sPLA_2$-IIA per mg of purified protein.

Statistical analysis

Statistical comparisons were performed with an unpaired, two-tailed Student's *t* test. P values less than 0.05 were considered statistically significant.

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REFERENCES

1. Kudo, I. and Murakami, M. (2002) Phospholipase A2 enzymes. *Prostaglandins. Other. Lipid. Mediat.* **68-69**, 3-58.
2. Menschikowski, M., Hagelgans, A. and Siegert, G. (2006) Secretory phospholipase A2 of group IIA: is it an offensive or a defensive player during atherosclerosis and other inflammatory diseases? *Prostaglandins. Other. Lipid. Mediat.* **79**, 1-33.
3. Crowl, R. M., Stoller, T. J., Conroy, R. R. and Stoner, C. R. (1991) Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. *J. Biol. Chem.* **266**, 2647-2651.
4. Oka, S. and Arita, H. (1991) Inflammatory factors stimulate expression of group II phospholipase A2 in rat cultured astrocytes. Two distinct pathways of the gene expression. *J. Biol. Chem.* **266**, 9956-9960.
5. Marcum, J. A. and Rosenberg, R. D. (1984) Anticoagulantly active heparin-like molecules from vascular tissue. *Biochemistry* **23**, 1730-1737.
6. Kaneider, N. C., Egger, P., Dunzendorfer, S. and Wiedermann, C. J. (2001) Syndecan-4 as antithrombin receptor of human neutrophils. *Biochem. Biophys. Res. Commun.* **287**, 42-46.
7. Parrillo, J. E. (1993) Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.* **328**, 1471-1477.
8. Bass, M. D. and Humphries, M. J. (2002) Cytoplasmic interactions of syndecan-4 orchestrate adhesion receptor and growth factor receptor signalling. *Biochem. J.* **368**, 1-15.
9. Delehedde, M., Lyon, M., Sergeant, N., Rahmoune, H. and Fernig, D. G. (2001) Proteoglycans: pericellular and cell surface multireceptors that integrate external stimuli in the mammary gland. *J. Mammary. Gland. Biol. Neoplasia.* **6**, 253-273.
10. Kaneider, N. C., Forster, E., Mosheimer, B., Sturn, D. H. and Wiedermann, C. J. (2003) Syndecan-4-dependent signaling in the inhibition of endotoxin-induced endothelial adherence of neutrophils by antithrombin. *Thromb. Haemost.* **90**, 1150-1157.
11. Bae, J. S. and Rezaie, A. R. (2009) Mutagenesis studies toward understanding the intracellular signaling mechanism of antithrombin. *J. Thromb. Haemost.* **7**, 803-810.
12. Menschikowski, M., Hagelgans, A., Hempel, U., Lattke, P., Ismailov, I. and Siegert, G. (2008) On interaction of activated protein C with human aortic smooth muscle cells attenuating the secretory group IIA phospholipase A2 expression. *Thromb. Res.* **122**, 69-76.
13. Bae, J. S. and Rezaie, A. R. (2010) Thrombin and activated protein C inhibit the expression of secretory group IIA phospholipase A(2) in the TNF-alpha-activated endothelial cells by EPCR and PAR-1 dependent mechanisms. *Thromb. Res.* **125**, e9-e15.
14. Sonoki, K., Iwase, M., Sasaki, N., Ohdo, S., Higuchi, S., Takata, Y. and Iida, M. (2008) Secretory PLA2 inhibitor indoxam suppresses LDL modification and associated inflammatory responses in TNFalpha-stimulated human endothelial cells. *Br. J. Pharmacol.* **153**, 1399-1408.
15. Hayashi, H., Tsuchiya, Y., Nakayama, K., Satoh, T. and Nishida, E. (2008) Down-regulation of the PI3-kinase/Akt pathway by ERK MAP kinase in growth factor signaling. *Genes Cells* **13**, 941-947.
16. Yao, R. and Cooper, G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**, 2003-2006.
17. Minshall, C., Arkins, S., Freund, G. G. and Kelley, K. W. (1996) Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *J. Immunol.* **156**, 939-947.
18. Ahmed, N. N., Grimes, H. L., Bellacosa, A., Chan, T. O. and Tsichlis, P. N. (1997) Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3627-3632.
19. Bae, J. S., Kim, Y. U., Park, M. K. and Rezaie, A. R. (2009) Concentration dependent dual effect of thrombin in endothelial cells via Par-1 and Pi3 Kinase. *J. Cell Physiol.* **219**, 744-751.
20. Nakano, T., Ohara, O., Teraoka, H. and Arita, H. (1990) Glucocorticoids suppress group II phospholipase A2 production by blocking mRNA synthesis and post-transcriptional expression. *J. Biol. Chem.* **265**, 12745-12748.
21. Pruzanski, W. and Vadas, P. (1991) Phospholipase A2--a mediator between proximal and distal effectors of inflammation. *Immunol. Today* **12**, 143-146.
22. Bradley, J. D., Dmitrienko, A. A., Kivitz, A. J., Gluck, O. S., Weaver, A. L., Wiesenhutter, C., Myers, S. L. and Sides, G. D. (2005) A randomized, double-blinded, placebo-controlled clinical trial of LY333013, a selective inhibitor of group II secretory phospholipase A2, in the treatment of rheumatoid arthritis. *J. Rheumatol.* **32**, 417-423.
23. Zeiher, B. G., Steingrub, J., Laterre, P. F., Dmitrienko, A., Fukiishi, Y. and Abraham, E. (2005) LY315920NA/S-5920, a selective inhibitor of group IIA secretory phospholipase A2, fails to improve clinical outcome for patients with severe sepsis. *Crit. Care Med.* **33**, 1741-1748.
24. Yamauchi, T., Umeda, F., Inoguchi, T. and Nawata, H. (1989) Antithrombin III stimulates prostacyclin production by cultured aortic endothelial cells. *Biochem. Biophys Res. Commun* **163**, 1404-1411.
25. Hoffmann, J. N., Vollmar, B., Romisch, J., Inthorn, D., Schildberg, F. W. and Menger, M. D. (2002) Antithrombin effects on endotoxin-induced microcirculatory disorders are mediated mainly by its interaction with microvascular endothelium. *Crit. Care Med.* **30**, 218-225.
26. Kaneider, N. C., Reinisch, C. M., Dunzendorfer, S., Romisch, J. and Wiedermann, C. J. (2002) Syndecan-4 mediates antithrombin-induced chemotaxis of human peripheral blood lymphocytes and monocytes. *J. Cell Sci.* **115**, 227-236.