Inhibitory effects of antithrombin on the expression of secretory group IIA phospholipase A2 in endothelial cells

Tae Hoon Kim1 & Jong-Sup Bae2,*
Departments of 1Herbal Medicinal Pharmacology, 2Herbal Pharmaceutical Engineering, College of Herbal Bio-Industry, Daegu Haany University, Gyeongsan 712-715, Korea

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 A2 (sPLA2-IIA). sPLA2-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 sPLA2-IIA in TNF-α-stimulated HUVECs. TNF-α potently upregulated the expression of sPLA2-IIA, and prior treatment of cells with AT inhibited the expression of sPLA2-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 sPLA2-IIA. These results show that AT effectively suppresses the upregulated sPLA2-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 A2 (PLA2) 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 PLA2 (sPLA2) 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, tumorogenesis, bacterial defense, fertilization, and phospholipid digestion in the gastrointestinal tract (1). Although the biological functions of sPLA2-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 sPLA2-IIA have been found in patients with severe inflammatory diseases (e.g. sepsis, septic shock, polytrauma), suggesting sPLA2-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 sPLA2-IIA production in human endothelial cells through its receptor, syndecan-4. In the present study, we investigated the effect of AT on sPLA2-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...
inhibitory effects on the expression of sPLA2-IIA in TNF-α-stimulated HUVECs.

RESULTS AND DISCUSSION

It is well known that TNF-α and other inflammatory cytokines mediate the expression of sPLA2-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 sPLA2-IIA (as occurs during inflammation), HUVECs were stimulated with TNF-α. Recently, we showed that TNF-α upregulated the expression levels of sPLA2-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 sPLA2-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 sPLA2-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 sPLA2-IIA by TNF-α in both supernatants and cell lysates, suggesting that TNF-α mediated upregulation of sPLA2-IIA was due to the new synthesis and secretion of the sPLA2-IIA.

A recent study showed that plasma-derived activated protein C (APC) effectively down-regulated the expression of sPLA2-IIA by interferon-γ (INF-γ) in human aortic smooth muscle cells (12). However, the effects of AT on sPLA2-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 sPLA2-IIA in TNF-α-stimulated Ea.hy926 cells (Fig. 2A, B) and primary HUVECs (Fig. 2C, D).

Next, we determined the IC50 of AT on the TNF-α-induced sPLA2-IIA stimulation (Table 1). The IC50 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 sPLA2-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 sPLA2-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-

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**Table 1. Inhibitory effect of AT on TNF-α induced sPLA2-IIA**

<table>
<thead>
<tr>
<th>AT on human EC</th>
<th>IC50 (U/ml)*</th>
</tr>
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<tbody>
<tr>
<td>Primary HUVEC</td>
<td>Supernatant 0.32†</td>
</tr>
<tr>
<td></td>
<td>Lysate 0.29</td>
</tr>
<tr>
<td>Ea.hy926</td>
<td>Supernatant 0.27</td>
</tr>
<tr>
<td></td>
<td>Lysate 0.24</td>
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*Each value represents the mean ± SD (n=3). † Each value expressed in units of AT.

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**Fig. 1.** Effect of TNF-α on the expression of sPLA2-IIA. Ea.hy926 cells were incubated with the indicated concentrations of TNF-α for four hours, followed by measurement of the expression level of sPLA2-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 sPLA2-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.