

Inhibitory Effect of an Urotensin II Receptor Antagonist on Proinflammatory Activation Induced by Urotensin II in Human Vascular Endothelial Cells

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Abstract

In this study, we investigated the effects of a selective urotensin II (UII) receptor antagonist, SB-657510, on the inflammatory response induced by UII in human umbilical vein endothelial cells (EA.hy926) and human monocytes (U937). UII induced inflammatory activation of endothelial cells through expression of proinflammatory cytokines (IL-1 β and IL-6), adhesion molecules (VCAM-1), and tissue factor (TF), which facilitates the adhesion of monocytes to EA.hy926 cells. Treatment with SB-657510 significantly inhibited UII-induced expression of IL-1 β , IL-6, and VCAM-1 in EA.hy926 cells. Further, SB-657510 dramatically blocked the UII-induced increase in adhesion between U937 and EA.hy926 cells. In addition, SB-657510 remarkably reduced UII-induced expression of TF in EA.hy926 cells. Taken together, our results demonstrate that the UII antagonist SB-657510 decreases the progression of inflammation induced by UII in endothelial cells.

Key Words: Urotensin II, Urotensin II receptor antagonist, Adhesion molecules, Cytokines, Tissue factor, Endothelial cells

INTRODUCTION

Atherosclerosis has been implicated as the cause of most cardiovascular diseases such as heart failure and myocardial infarction. Atherosclerosis is associated with endothelial cell injury and/or dysfunction, which results in lipid and leukocyte deposition in the arterial wall (Ross, 1993; Harrison and Ohara, 1995). Endothelial cells are the first cell line to be damaged during atherosclerosis, and endothelial dysfunction is a key event in the development of atherosclerotic lesions and remodeling of the heart after injury (Ross, 1993). Therefore, endothelial cell dysfunction can be regarded as an early marker for cardiovascular disease (Chatterjee *et al.*, 2008).

Urotensin II (UII), a cyclic peptide of 11 amino acids, has been identified as a potential mediator in the pathogenesis of cardiovascular diseases. UII is highly expressed in many tissues, including the heart and vasculature (Douglas *et al.*, 2002). The expression of UII is positively correlated with the severity of heart function and atherosclerosis. The expression of UII is increased in the cardiac tissue of patients with congestive heart failure (CHF) (Douglas *et al.*, 2002) and in the

endothelial cells, smooth muscle cells, and infiltrating macrophages in atherosclerotic lesions of human coronary arteries (Bousette *et al.*, 2004; Hassan *et al.*, 2005). The plasma UII levels are high in patients with atherosclerosis and coronary artery disease (Suguro *et al.*, 2007; Loirand *et al.*, 2008). Additionally, UII increases the expression of inflammatory mediators (adhesion molecules and tissue factor [TF]), which are well-known risk factors for the development of atherosclerosis or thrombosis in human coronary endothelial cells (Cirillo *et al.*, 2008). Taken together, the increased expression of UII may contribute to the pathogenesis of atherosclerosis. In fact, UII gene deletion or UII receptor (UT) blockade reduces initiation and progression of atherosclerosis *in vivo* (You *et al.*, 2012). Therefore, inhibition of UII signaling has been suggested as a potential therapeutic target for atherosclerosis.

Recent studies have shown that a highly specific UT antagonist, SB-657510, inhibits the progression of high-fat diet-induced atherosclerosis and diabetes-associated atherosclerosis *in vivo* (Papadopoulos *et al.*, 2009; You *et al.*, 2012; Watson *et al.*, 2013). However, the anti-inflammatory effects of SB-657510 at a cellular level have not been evaluated to date.

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In this study, we investigated the role of UII and UT antagonist SB-657510 in UII-induced inflammatory responses in human vascular endothelial cells.

MATERIALS AND METHODS

Cell culture

EA.hy926 cells, a human umbilical vein endothelial cell line, were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. U937 cells, a human monocyte cell line, were maintained in Roswell Park Memorial Institute medium (Gibco-BRL) supplemented with 10% FBS and antibiotics. Cells were incubated in an atmosphere of 95% air and 5% CO₂ at 37°C. EA.hy926 cells were pretreated with SB-657510 (Sigma-Aldrich, St Louis, MO, USA) for 30 min. Subsequently, the cells were treated with 100 nM UII (Sigma-Aldrich) in the presence or absence of SB-657510.

Cell-cell adhesion assay

EA.hy926 cells (5×10⁴ cells/well) were seeded in 24-well plates and grown to 80-85% confluence. Then, the EA.hy926 cells were treated with 100 nM UII in the presence or absence of SB-657510. After washing, U937 monocytes (6×10⁵ cells/well) were added to the UII-treated EA.hy926 cells. After incubation for 1 h, non-adherent U937 monocytes were removed through washing with PBS. Cells were counted in 5 fields per well using an optical microscope. Adherent cells were calculated by expressing the numbers of adherent U937 monocytes as a percentage of total EA.hy926 cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of adhesion molecules, cytokines, and TF mRNA were measured using RT-PCR as previously described (Lee *et al.*, 2011). cDNAs were synthesized from total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Then, the cDNAs were amplified by PCR using gene-specific primers. All PCR primers are listed in Table 1. Relative quantification of mRNA was performed by densitometry using QuantityOne software (Biorad Laboratories, Philadelphia, PA, USA).

Western blot analysis

Protein expression of TF was measured using western blot analysis as previously described (Lee *et al.*, 2012). Cells were lysed using lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 150 mmol/L NaCl, 0.25% Na-deoxycholate, 2 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L PMSF, 10 μg/mL of aprotinin, and 10 μmol/L leupeptin). Lysates were subsequently centrifuged at 14,000 rpm for 15 min and supernatants were collected. Then, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reacted with anti-tissue factor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin antibody (Sigma-Aldrich). After probing with horseradish peroxidase (HRP)-conjugated secondary antibody, proteins were visualized using LAS 1000 (Fuji Photo Film, Tokyo, Japan). Densitometric analyses were performed using QuantityOne software (Biorad Laboratories).

Statistical analysis

All data were expressed as the mean ± SEM from at least 3 different experiments. The comparisons were performed using Student's *t*-tests. A *p* value of <0.05 was considered statistically significant.

Table 1. Oligonucleotide sequences of primers for RT-PCR

Gene	Accession number	Sequences (5'-3')	Tm (°C)
Actin	NM_001101	CCC AGA TCA TGT TTG AGA CCT ATG TCA CGC ACG ATT TCC C	57
ICAM-1	NM_000201	CGA CTG GAC GAG AGG GAT TG TTA TGA CTG CGG CTG CTA CC	57
IL-1β	NM_000576	TAC CTG AGC TCG CCA GTG AAA GGA AGA CAC AAA TTG CAT GG	57
IL-6	NM_000600	AGA GTA GTG AGG AAC AAG CC TAC ATT TGC CGA AGA GCC CT	57
IL-8	NM_000584	TGT TCC ACT GTG CCT TGG TT CTG GCAATG ACA AGA CTG GG	57
PECAM-1	NM_000442	AGC CAA CTT CAC CAT CCA GAA CGT TGT TGG AGT TCA GAA GTG	57
Tissue factor	NM_001993	TGC TAT ATT GCA CTG TGA CCG CAC CTG AGG TCA GGA ATT CAA	60
TNF-α	NM_000594	AGG CCA AGC CCT GGT ATG AGC CAC AGG GCA ATG ATC CCA AAG TAG	57
Urotensin II	NM_021995	CTT TCA ACT CTC AGC ACC TCA CCT AGT TTT TCT CCA CAC TGT T	57
Urotensin receptor	NM_018949	CGC AAC CCT CAA CAG CTC CT CCT TGG TGA CGT AGG TGG CC	57
VCAM-1	NM_001078	ACG AAC ACT CTT ACC TGT GCA AAT TTA GCT CGG CAA ACA AGA	57

RESULTS

Effect of SB-657510 on UII-induced attachment of monocytes to endothelial cells

One of the earliest events in inflammation is binding and infiltration of monocytes through the endothelium. Therefore, monocyte-endothelial interactions are important in the development of atherosclerosis (Grenon *et al.*, 2012). To determine whether SB-657510 inhibits a primary process of inflammation, we performed cell-cell adhesion assay. Treatment of EA.hy926 cells with 100 nM UII significantly increased the number of U937 attached to the activated endothelial cells in a time-dependent manner (Fig. 1A). SB-657510 treatment inhibited the adhesion of monocytes to UII-stimulated EA.hy926 cells ($544.4 \pm 117.7\%$) in a dose-dependent manner (Fig. 1B). In particular, 1 μM SB-657510 (non-cytotoxic dose, data not shown) dramatically decreased the attachment of U937 to that of the control level ($148.6 \pm 54.8\%$). This finding indicates that SB-657510 inhibits the attachment of monocytes to endothelial cells by blocking the stimulation of endothelial cells with UII.

Effect of SB-657510 on UII-induced expression of adhesion molecules

As shown in Fig. 2A, we found that UII and UT mRNA were expressed in EA.hy926 cells. Cell-cell interaction is regulated by the expression of adhesion molecules on the endothelial cells, which occurs as a result of inflammatory activation of the endothelium (Grenon *et al.*, 2012). To evaluate the effect of SB-657510 on the expression of adhesion molecules in endothelial cells, we investigated the mRNA expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell

adhesion molecule 1 (PECAM-1) in EA.hy926 cells. Although the expressions of ICAM-1 and PECAM-1 were not changed by 100 nM UII, the expression of VCAM-1 was increased by UII (Fig. 2B). And the UII-induced 4.8-fold increase in VCAM-1 expression was markedly inhibited to 2.3-fold upon treatment with 1 μM SB-657510 (Fig. 2C). This finding indicates that SB-657510 blocks VCAM-1 expression induced by UII in endothelial cells.

Effect of SB-657510 on UII-induced expression of cytokines

Proinflammatory cytokines are well known to regulate the expression of adhesion molecules in various cells (Chen *et al.*, 2001; Kwon *et al.*, 2007). To examine the effect of SB-657510 on the expression of cytokines in endothelial cells, we investigated the mRNA expression of cytokines (IL-1 β , IL-6, IL-8, and TNF- β) in EA.hy926 cells. The expression of IL-8 and TNF- β was unchanged by 100 nM UII. However, the expression of IL-1 β and IL-6 increased after treatment with UII (Fig. 3A). The UII-induced 8.9 fold increase in the expression of IL-1 β and 2.8 fold increase in the expression of IL-6 were markedly inhibited to 2.8 fold and 1 fold, respectively, upon treatment with 1 μM SB-657510 (Fig. 3B). This finding indicates that SB-657510 blocks IL-1 β and IL-6 expression induced by UII in endothelial cells.

Effect of SB-657510 on UII-induced expression of TF

Inflammatory mediators activate induction of the TF by the endothelium, which could contribute to both hemostasis and the formation of an occlusive thrombus (Lwaleed *et al.*, 2007), and, the TF molecule has strong proinflammatory properties, which cause arthritis (Bokarewa *et al.*, 2002). Therefore, we investigated the effect of SB-657510 on the mRNA expres-

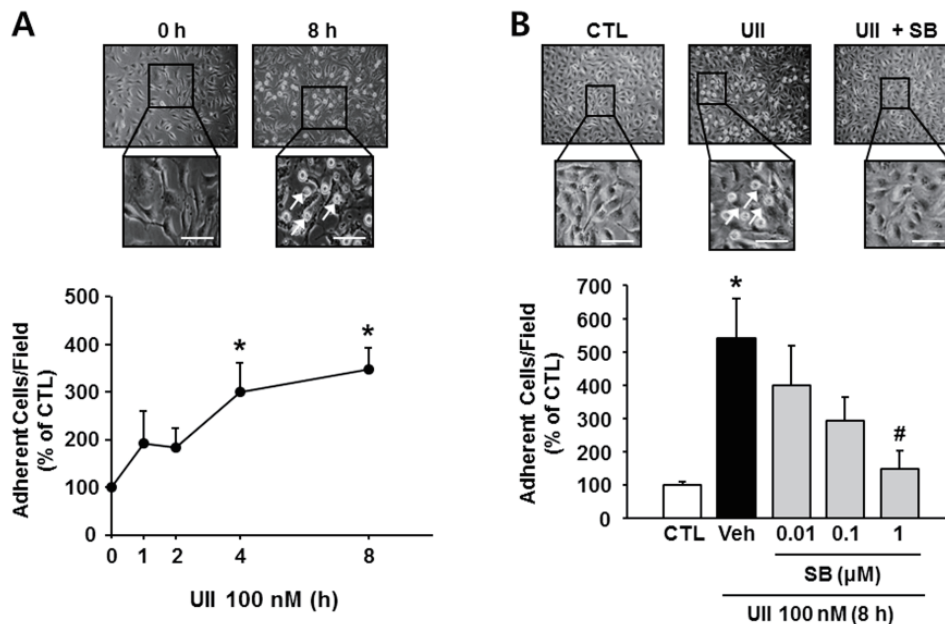


Fig. 1. Effect of SB-657510 (SB) on uterotensin II (UII)-induced attachment of monocytes to endothelial cells. After treatment with 100 nM UII for the indicated times in the presence or absence of SB, EA.hy926 cells were incubated with U937 monocytes for 1 h. Representative images and quantitative analysis are shown in top and bottom, respectively. (A) Adhesion assay after UII treatment. Arrow, attached U937 monocyte; scale bar, 100 μm . * $p < 0.05$ vs. 0 h. $n = 3$. (B) Adhesion assay after UII and SB treatment. Arrow, attached U937 monocyte, scale bar, 100 μm . * $p < 0.05$ vs. control (CTL), # $p < 0.05$ vs. vehicle (Veh). $n = 3$.

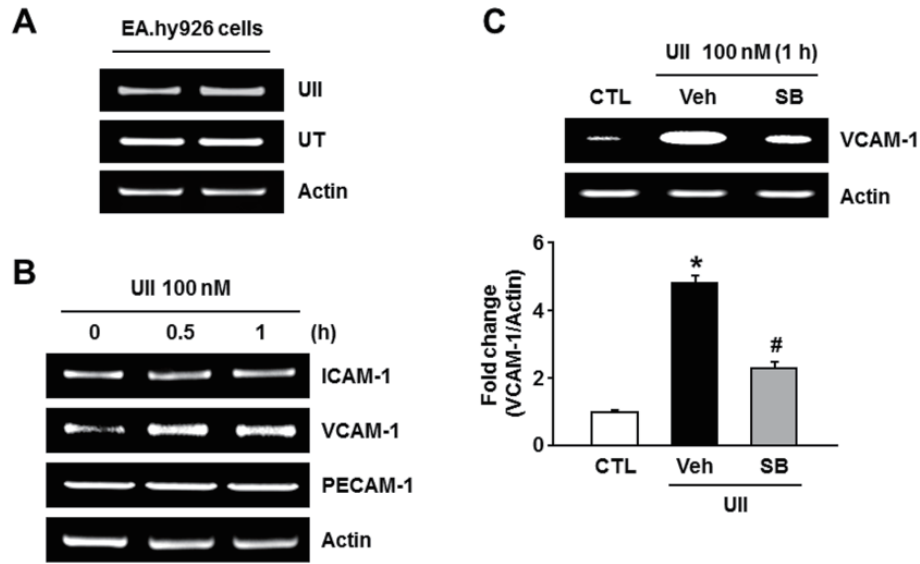


Fig. 2. Effect of SB-657510 (SB) on urotensin II (UII)-induced expression of adhesion molecules. (A) RT-PCR of UII and UII receptor (UT) in EA.hy926 cells. n=4. (B) RT-PCR of adhesion molecules after UII treatment. EA.hy926 cells were treated with 100 nM UII for 0.5 or 1 h. n=4. (C) RT-PCR of VCAM-1 after UII and SB treatment. After pretreatment with 1 μ M SB, cells were treated with 100 nM UII for 1 h in the presence or absence of SB. Quantitative analysis is shown in the bottom. * p <0.05 vs. control (CTL), # p <0.05 vs. vehicle (Veh). n=4.

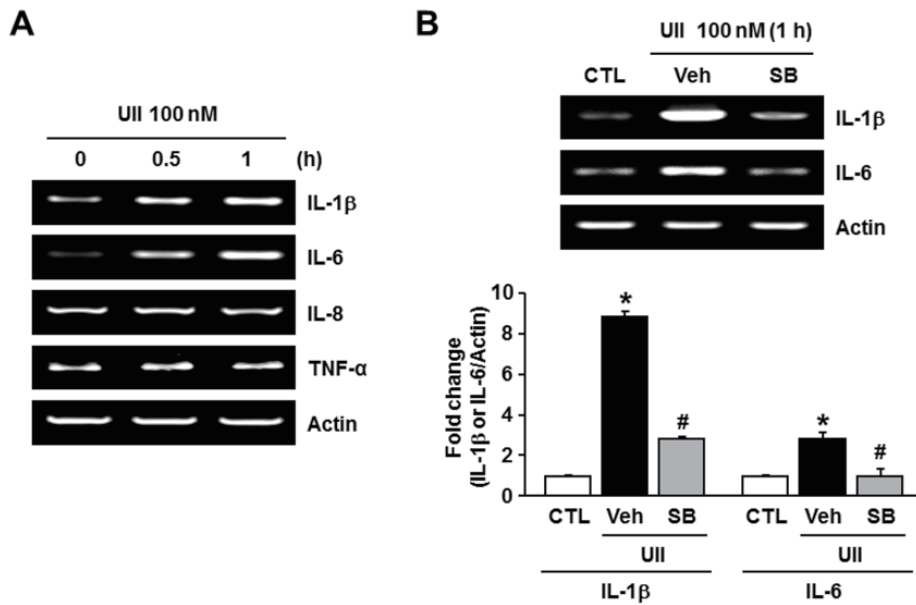


Fig. 3. Effect of SB-657510 (SB) on urotensin II (UII)-induced expression of inflammatory cytokines. (A) RT-PCR of inflammatory cytokines after UII treatment. EA.hy926 cells were treated with 100 nM UII for 0.5 or 1 h. n=4. (B) RT-PCR of IL-1 β and IL-6 after UII and SB treatment. After pretreatment with 1 μ M SB, cells were treated with 100 nM UII for 1 h in the presence or absence of SB. Quantitative analysis is shown in the bottom. * p <0.05 vs. control (CTL), # p <0.05 vs. vehicle (Veh). n=4.

sion of TF in endothelial cells. The mRNA expression of TF increased after treatment with 100 nM UII (Fig. 4A). And the UII-induced 3.3 fold increase in the mRNA expression of TF was significantly inhibited to 1.4 fold upon treatment with 1 μ M SB-657510 (Fig. 4B). This finding indicates that SB-657510 blocks IL-1 β and IL-6 expression induced by UII in endothelial

cells. Next, we investigated the effect of SB-657510 on the protein expression of TF in endothelial cells. Treatment with 100 nm UII induced a time-dependent increase in the expression of TF protein similar to the results of mRNA expression (Fig. 4C). And treatment with 1 μ M SB-657510 remarkably decreased the UII-induced protein expression of TF to that of the

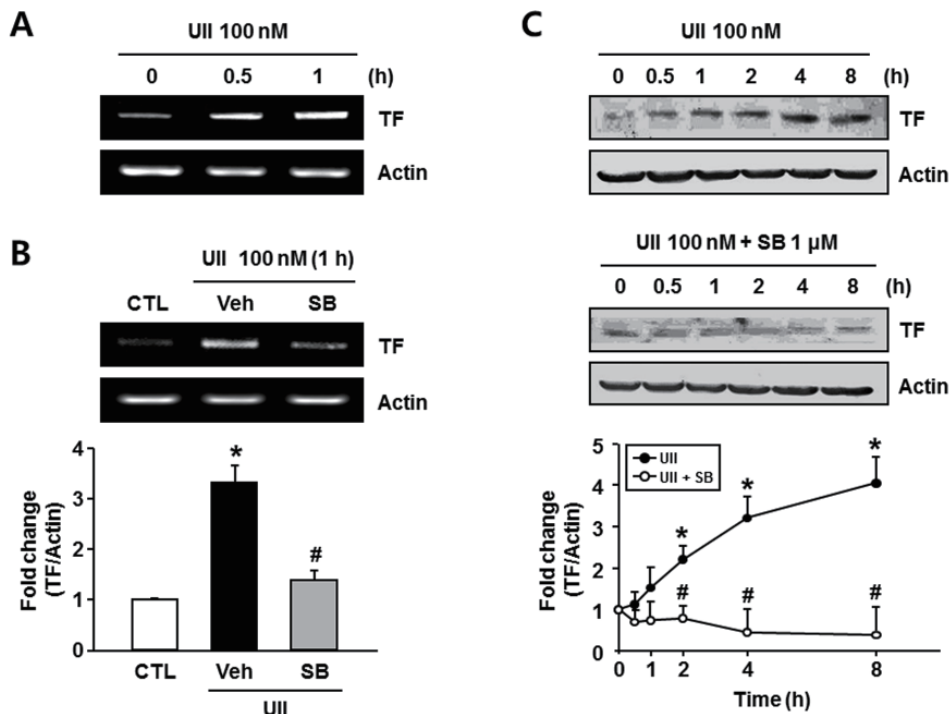


Fig. 4. Effect of SB-657510 (SB) on uterins II (Ull)-induced expression of tissue factor (TF). (A) RT-PCR of TF after Ull treatment. EA.hy926 cells were treated with 100 nM Ull for 0.5 or 1 h. $n=4$. (B) RT-PCR of TF after Ull and SB treatment. After pretreatment with 1 μ M SB, cells were treated with 100 nM Ull for 1 h in the presence or absence of SB. Quantitative analysis is shown in the bottom. * $p<0.05$ vs. control (CTL), # $p<0.05$ vs. vehicle (Veh). $n=4$. (C) Western blots of TF after Ull and SB treatment. After pretreatment with 1 μ M SB, cells were treated with 100 nM Ull for the indicated times in the presence or absence of SB. Quantitative analysis is shown in the bottom. * $p<0.05$ vs. 0 h, # $p<0.05$ vs. Ull. $n=4$.

control level. This finding indicates that SB-657510 blocks the expression of TF induced by Ull in endothelial cells.

DISCUSSION

This study shows that SB-657510, a selective UT antagonist, exerts anti-inflammatory effects by inhibiting Ull-induced upregulation of inflammatory mediators such as adhesion molecules, cytokines, and TF in human vascular endothelial cells.

Inflammation is a well-known risk factor for the development of atherosclerosis and plays a pivotal role in all stages of atherosclerosis (Steinberg, 2002). Several experimental studies and clinical observations have confirmed that a variety of inflammatory mediators such as adhesion molecules, cytokines, and TF, participate in the progression of atherosclerotic lesions (Tremoli *et al.*, 1999; Kleemann *et al.*, 2008). Adhesion molecules, including VCAM-1, ICAM-1, PECAM-1, and E-selectin, play pivotal roles in mediating the migration of leucocytes to the site of inflammation and its subsequent adhesion to endothelial cells, which is one of the major mechanisms underlying the pathogenesis of atherosclerosis (O' Brien *et al.*, 1996; Price and Loscalzo, 1999; Libby, 2002). In particular, VCAM-1 is expressed by endothelial cells in atherosclerotic lesions, and it promotes the adhesion of monocytes on the vascular endothelium (Cybulsky *et al.*, 2001; Tedgui and Mallat, 2006). Furthermore, deletion of VCAM-1 reduces atherosclerotic lesions in hyperlipidemic mouse models (Ramos *et al.*, 1999).

These studies indicate that expression of VCAM-1 is essential for the inflammatory process in atherosclerosis lesions (Galkina and Ley, 2007). In the present study, Ull increased VCAM-1 mRNA expression but not ICAM-1 and PECAM-1 mRNA expression in human umbilical vein endothelial cells. On the other hand, not only VCAM-1 but also ICAM-1 was increased by Ull in human coronary endothelial cells (Cirillo *et al.*, 2008). These distinct results can be explained by differences in cell-type and species. In this study, SB-657510 inhibited the function of VCAM-1 induced by Ull, shown by its inhibition of attachment between monocytes and endothelial cells. These results suggest that SB-657510 has anti-inflammatory effects by reducing Ull-induced VCAM-1 expression and attachments between monocytes and endothelial cells.

Proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8, are key players in the pathogenesis of atherosclerosis and regulate the expression of adhesion molecules (Tedgui and Mallat, 2006; Kleemann *et al.*, 2008). Several studies showed that TNF- α and IL-1 β induce VCAM-1 expression in vascular endothelial cells (Haraldsen *et al.*, 1996; Min *et al.*, 2005). At a cellular level, Ull stimulated mRNA expressions of IL-1 β and IL-6, and these increases were inhibited by SB-657510. A recent study of You *et al.* showed that SB-657510 decreased the serum level of other cytokines, including tissue inhibitor of metalloproteinase-1, M-CSF, and MIG in apoE KO mice (You *et al.*, 2012). In that study, they further demonstrated that SB-657510 inhibited aortic ERK1/2 activation, suggesting its ameliorating effect on the experimental atherosclerosis

(You *et al.*, 2012). In this point of view, it will be meaningful to extend our study to confirm those parameters at the vascular smooth muscle cell level.

Various studies suggest that TF, a key initiator of the coagulation cascade, is involved in the pathogenesis of atherosclerosis by promoting thrombus formation (Steffel *et al.*, 2006). The expression of TF in endothelial cells can be induced by cytokines such as TNF- α and IL-1 β , which could promote a prothrombotic phenotype of the endothelium and finally lead to the occurrence of acute coronary syndromes (Steffel *et al.*, 2006; Cirillo *et al.*, 2008). A recent study showed that Ull induces the expression of TF in human coronary artery endothelial cells, which indicate the pro-atherothrombotic effects of Ull (Cirillo *et al.*, 2008). Consistent with findings reported in previous study, our study showed that Ull increased the expression of TF in human umbilical vein endothelial cells, and the Ull-induced TF expression was significantly suppressed by SB-657510. Thus, our results suggest that SB-657510 has the potential to block Ull-induced endothelial pro-thrombotic phenotype through inhibition of TF expression.

In conclusion, the present study showed that SB-657510 inhibits Ull-induced expression of VCAM-1, IL-1 β , IL-6, and TF in human vascular endothelial cells, supporting the *in vivo* anti-atherosclerotic effect of SB-657510 reported previously. Our results will provide insights in understanding the protective role of SB-657510 against atherosclerosis.

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