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Salvianolic Acid B Attenuates Plasminogen Activator Inhibitor Type 1 Production in TNF-α Treated Human Umbilical Vein Endothelial Cells

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Abstract Plasminogen activator inhibitor type 1 (PAI-1), which plays a role in the development of atherosclerosis, is produced by endothelial cells following stimulation with various inflammatory cytokines such as tumor necrosis factor (TNF-α). In the present study, we investigated the effects of a potent water-soluble antioxidant, salvianolic acid B (SalB; derived from the Chinese herb, *Salvia miltiorrhiza*), on the expression of PAI-1 in TNF-α-treated human umbilical vein endothelial cells (HUVECs). We found that SalB inhibited TNF-α-induced PAI-1 mRNA production and protein secretion in HUVECs. Treatment with SalB (0.05 and 0.15 μM) notably attenuated TNF-α induced expression of PAI-1 to 90.5% and 74.6%, respectively, after 12 h, and to 75.1% and 64.2%, respectively, after 18 h. We also observed a dose-dependent decrease in PAI-1 protein production in the presence of SalB. We then used pathway inhibitors to investigate which step of the TNF-α induced signaling pathway was targeted by SalB. We found that the c-Jun N-terminal kinase (JNK) inhibitor, SP600125, increased the inhibitory effects of SalB on TNF-α-induced PAI-1 secretion, whereas the nuclear factor-κB (NF-κB) inhibitor, emodin, and the extracellular signal-regulated kinase (ERK) inhibitor, PD98059, did not. A gel shift assay further showed that SalB inhibited the TNF-α-activated NF-κB and AP-1 DNA binding activities in a dose-dependent manner. Collectively, these results indicate that the NF-κB and ERK-AP-1 pathways are possible targets of SalB in the regulation of TNF-α-stimulated PAI-1 production in HUVECs. J. Cell. Biochem. 96: 109–116, 2005.

Key words: salvianolic acid B; plasminogen activator inhibitor type 1; endothelial cells; tumor necrosis factor-α

The vascular endothelium plays a pivotal role in initiation and control of fibrinolysis through the synthesis of plasminogen activator inhibitor type-1 (PAI-1) and tissue-type plasminogen activator (t-PA). PAI-1 is the primary inhibitor of tissue-type and urokinase-type plasminogen activators; impaired fibrinolytic activity is often the consequence of an increase in circulating levels of PAI-1. Therefore, PAI-1 is considered to play an integral role in controlling the fibrinolytic system [Huber, 2001; Fay, 2004].

Atherosclerosis is a chronic inflammatory process characterized by macrophage and monocyte infiltration through the vessel wall, as well as intimal hyperplasia due to the migration and proliferation of vascular smooth muscle cells after endothelial injury or degeneration by oxidative stress [Davignon and Ganz, 2004; Stocker and Keaney, 2004]. PAI-1 is thought to play a crucial role in the development of atherosclerosis, as its expression is upregulated in patients with hypertension, type 2 diabetes, insulin resistance, and hypertriglyceridemia, which are known to accelerate atherosclerosis [Grant, 2003; Jovin and Muller-Berghaus, 2004]. Previous studies emphasizing the pathophysiological roles of PAI-1 seem to suggest that modulation of endothelial PAI-1 expression may represent an attractive therapeutic approach for atherosclerosis [Juhan-Vague et al., 2003].

Salvia miltiorrhiza (SM) has been used in traditional Chinese medicine for thousands of years and is now widely used for treatment of cardiovascular diseases. Previous studies have demonstrated that SM is an anti-inflammatory,

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anti-oxidative, and apoptosis-inducing plant. For example, SM reduces myocardial infarct size, increases coronary blood flow, and protects the heart against ischemia-reperfusion injury [Lei and Chiou, 1986; Lay et al., 2003]. A water-soluble extract from SM has been shown to reduce atherosclerosis in cholesterol-fed rabbits [Wu et al., 1998; Chen et al., 2001b]. Salvianolic acid B (SalB), a pure water-soluble compound extracted from SM, has been reported to exert a protective action against tumor necrosis factor (TNF-α) injury in human aortic vascular endothelial cells by reducing expression of cell adhesion molecules and cell adhesion. As SalB is thought to inhibit the initiation and progression of atherosclerosis [Chen et al., 2001a], and elevation of PAI-1 expression is related to atherosclerotic lesions, we herein examined whether treatment with SalB modulates TNF-α-induced PAI-1 synthesis.

MATERIALS AND METHODS

Reagents

SalB and emodin (authentic standards) were purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Special precautions were taken to avoid direct exposure of SalB to light and air during experiments. SalB was dissolved in warm culture medium just before incubation with the human umbilical vein endothelial cells (HUVECs). Recombinant human TNF-α was from Sigma (Steinheim, Germany). SP600125 was from Calbiochem (Damstardt, Germany). PD98059, the ImProm-II™ Reverse Transcription System, the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS), the PCR system, and the Gel shift core system were purchased from Promega (Madison, WI). The PAI-1 and t-PA Elisa kits were from Technoclone Gmbh (Wien, Austria).

Cell Culture

HUVECs were obtained from Cascade Biologics (Portland, OR) as cryopreserved primary cultures, and grown in culture flasks (Costar) in endothelial cell growth medium M200 (Cascade Biologics) supplemented with 2% LSGS (low serum growth supplement; Cascade Biologics), according to the Cascade Biologics' recommended protocol. The growth medium was changed every other day until cells reached confluence. Cells of passages 3 and 4 were grown in monolayers at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and used for experiments at >80% confluency.

Twenty-four hours before experiments, the total medium was removed and replaced with LSGS-free medium containing 0.4% FBS. For experiments, HUVECs were cultured in medium containing 0.4% FBS with or without the agents in the presence of 10 ng/ml TNF-α. After 12 or 18 h, the cell culture supernatants were collected from each well and assayed.

Assessment of Cell Viability

For evaluation of cytotoxicity, cells were seeded at 5,000 cells/well into 96-well culture plates (Costar) and grown for 48 h. The cells were incubated with various concentrations of the agents in serum-free M200 medium containing 0.4% FBS for 24 h, and then 20 μl of MTS was added to each well and further incubated at 37°C for 2 h. The absorbance of the solubilized formazan was read at 490 nm using a Victor™ 1420 Multilabel Counter (Wallac, Turku, Finland). Cells incubated in control media were considered 100% viable.

Antigen Assays for PAI-1 and t-PA

The levels of PAI-1 and t-PA antigens in HUVEC culture supernatants were determined by enzyme-linked immunosorbent assay methods, according to the manufacturer's recommended protocol.

RNA Isolation and Reverse Transcript Polymerase Chain Reaction (RT-PCR)

For multiplex semi-quantitative RT-PCR analysis, total RNA was isolated from HUVECs using the Trizol reagent (Invitrogen). Total RNA (2 μg) was reverse-transcribed into cDNA using an oligo(dT) primer, and then amplified with specific primers. The following primers with the predicted size were used for amplification: PAI-1(347 bp) 5′-CCACTTC-TTCAGGCTGTTCC-3′ (forward) and 5′-GCA-GTTCCAGGATGTCGTAG-3′ (reverse) and GAPDH (452 bp) 5′-ACCACAGTCCATGCCAT-CAC-3′ (forward) and 5′-TCCACCACCCT-GTTGCTGTA-3′ (reverse). The cycle number was determined from a linear amplification curve as being within the linear amplification range. The PCR conditions were as follows: 29 cycles of 94°C for 30 s, 58°C for 30 s, and 30 s at 70°C. The PCR products were subjected to 2% agarose gel electrophoresis. Quantitative data

normalized to GAPDH were obtained from a densitometer and analyzed with the included Quantity One 4.4.0 software (BIO-RAD).

Nuclear Protein Extract and Western Blot

Nuclear proteins were extracted and quantified, as described [Wattel et al., 2004]. Protein samples (20 μg) were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell Biosciences, Keene, NH) at room temperature. Membranes were preincubated for 2 h at room temperature with PBS-Tween 20 (0.05%)/2% skim milk, and then incubated for 1 h at room temperature with rabbit anti-human-nuclear factor-κB (NF-κB) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology). Antigen detection was performed with an Enhanced Chemiluminescence (ECL) kit (Santa Cruz Biotechnology), and blots were exposed to XBT-1 film (Kodak, Shantou, China).

Fluorescent Electrophoretic Mobility Shift Assay (fEMSA)

fEMSA was performed according to the method of Ruscher et al. [2000] with minor modifications. The double-stranded gel-shift oligonucleotides for NF-κB, and AP-1 were end-labeled with Cy5-end-labeling (Amersham Pharmacia Biotech).The binding reaction was performed for 20 min at 25°C in a total volume of 10 μl of binding buffer that contained 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol, 0.5 μg of double stranded poly (dI-dC). DNA–protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 250 V for 30 min in 0.5 × Tris borate-EDTA (1 × Trisborate-EDTA that contains 90 mM Tris-borate, pH 8.3, 2mM EDTA) at 4°C. After electrophoresis, gels were scanned with a Typhoon 9410 scanner (Pharmacia) at an excitation wavelength of 630 nm and an emission wavelength of 670 nm.

Statistical Analysis

All values are expressed as the mean ± standard errors of mean (SEM) of three independent determinations. Statistical analyses were performed with analysis of variance (ANOVA) and Tukey tests using the OriginPro 7.5 software (Originlab, Northampton, MA).

RESULTS

Effect of SalB on PAI-1 mRNA Expression and Protein Secretion

To examine the direct effects of SalB on TNF-α induced PAI-1 secretion, HUVECs were cultured in media with or without SalB (0.017, 0.05, or 0.15 μM) in the presence of TNF-α (10 ng/ml) for 12 and 18 h. As shown in Figure 1, SalB dose dependently inhibited TNF-α induced PAI-1 secretion from HUVECs. The decrease became

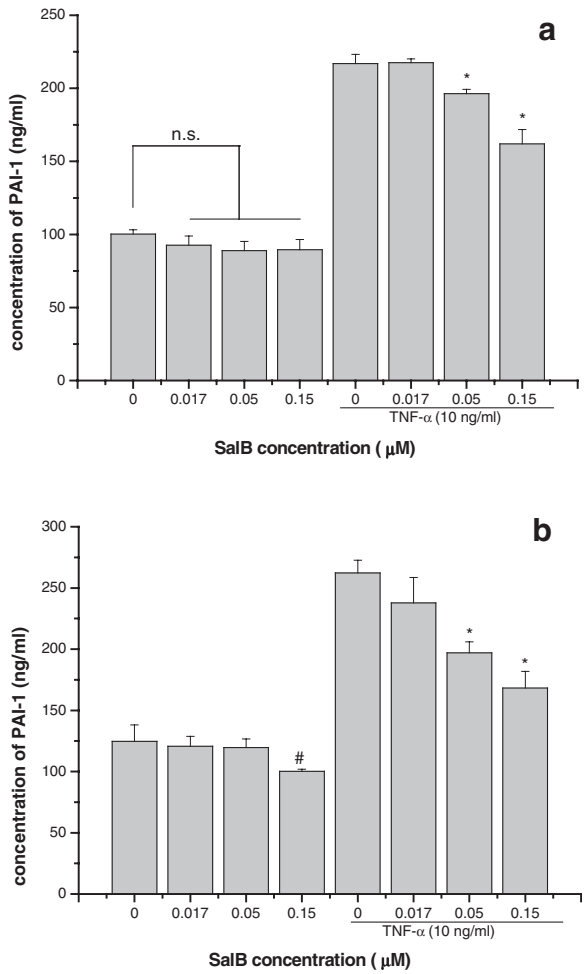

Fig. 1. Effects of various concentrations of SalB on PAI-1 secretion in HUVECs stimulated with TNF-α. HUVECs were cultured with or without SalB in the presence of TNF-α (10 ng/ml) for 12 h (**a**) and 18 h (**b**), and PAI-1 concentration in the culture medium was examined. The data represent the mean ± SEM (n = 3; $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. TNF-α alone; n.s., not significant).

significant ($P < 0.05$) at a PAI-1 dose of 0.05 μM. SalB also inhibited basal level of PAI-1 secretion at 18 h.

To investigate whether SalB inhibits PAI-1 production at the mRNA expression level in HUVECs, we performed a RT-PCR analysis on RNA isolated from HUVECs cultured in the presence of TNF-α with or without SalB. As shown in Figure 2, TNF-α induced a marked increase in PAI-1 mRNA, whereas treatment with SalB dose dependently inhibited TNF-α-induced PAI-1 mRNA expression. As a control, MTS analysis of endothelial cell viability revealed no significant changes in cell viability across a range of SalB concentrations from 0.05 to 5 μM.

Effects of TNF-α and SalB on t-PA Secretion

Because the balance between PAI-1 and t-PA determines fibrinolytic activity, we investigated the effects of TNF-α and SalB on t-PA production from HUVECs. TNF-α modestly decreased t-PA production at 18 h; this decrease was not significantly altered by treatment with SalB (Fig. 3).

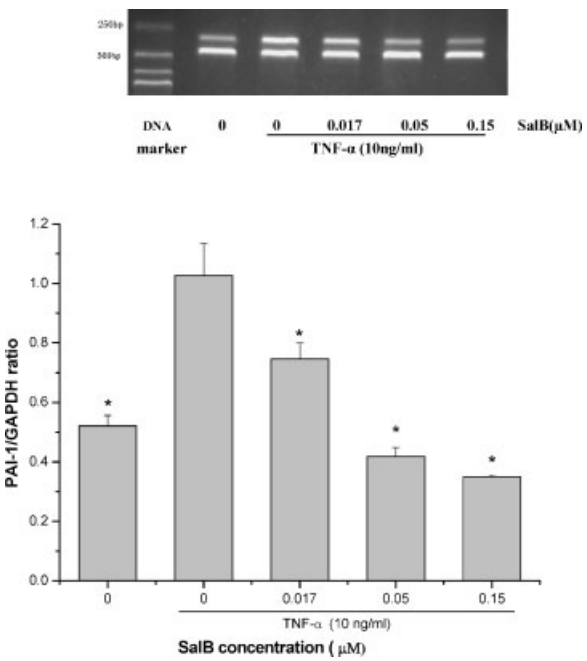

Fig. 2. RT-PCR shows the dose-dependent effect of SalB on PAI-1 mRNA expression in TNF-α-induced HUVECs after 18 h. Data are expressed as mean ± SEM (n = 3). Asterisks indicate a statistically significant difference when compared to results from TNF-α stimulated cells in the absence of SalB (*$P < 0.05$).

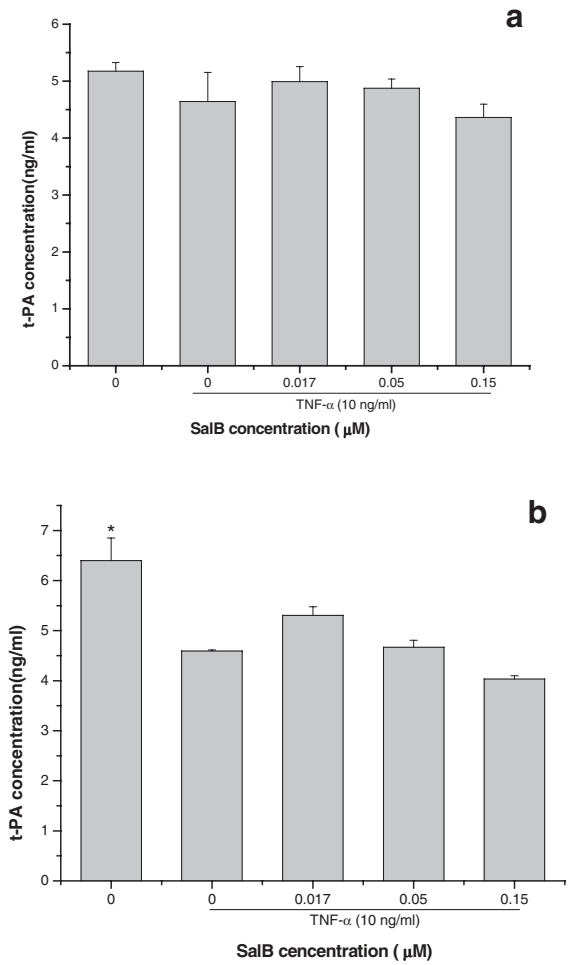

Fig. 3. Effects of various concentrations of SalB on t-PA secretion in HUVECs stimulated with TNF-α. HUVECs were cultured with or without SalB in the presence of TNF-α (10 ng/ml) for 12 h (**a**) and 18 h (**b**). The data represent the mean ± SEM (n = 3; #$P < 0.05$ vs. control; *$P < 0.05$ vs. TNF-α alone; n.s., not significant).

Effects of Signal Transduction Inhibitors on Basal and TNF-α-Induced PAI-1 Secretion in the Presence or Absence of SalB

Both NF-κB-dependent and ERK-dependent pathways are involved in TNF-α-induced PAI-1 production [Hamaguchi et al., 2003]. To clarify the target of SalB in the signal transduction pathways leading to TNF-α-induced PAI-1 expression, we investigated the effects of three signal transduction inhibitors, emodin (a NF-κB inhibitor), PD98059 [an extracellular signal-regulated kinase (ERK) inhibitor], and SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor] on TNF-α-induced PAI-1 expression in the presence or absence of SalB. We first

determined the effect of increasing doses of each inhibitor on HUVEC viability, and selected appropriate non-cytotoxic doses. To confirm the specificity of the inhibitors, we checked the cell viability for all combinations of agents by MTS assay. We found that the inhibitors combined with TNF-α and/or SalB as used in this study has no effect on HUVEC viability. We then performed the experiments that showed that PD98059 and emodin inhibited TNF-α-induced PAI-1 antigen accumulation in the culture medium, which is concordant with the results of a previous study [Hamaguchi et al., 2003]. We also observed that SP600125 did inhibit TNF-α-induced PAI-1 secretion. As shown in Figure 4a, neither PD98059 nor emodin showed any additional inhibitory effects in the presence of SalB. In contrast, SP600125 the inhibitory effects of SP600125 at low and high doses were essentially additive with those of SalB (Fig. 4b). Emodin, PD98059 or SP600125 showed no inhibitory effects on the TNF-α-induced PAI-1 secretion.

Effect of SalB on TNF-α-Induced NF-κ p65 and AP-1 Activation

To determine whether activation of NF-κB and/or AP-1 was involved in the effect of SalB on TNF-α-stimulated PAI-1 expression, we measured NF-κB and AP-1 DNA-binding activities in HUVEC nuclear extracts, and also measured the amount of NF-κB p65 nuclear translocation. TNF-α-stimulated HUVECs showed a marked increase in nuclear translocation of NF-κB p65, as measured by Western blot (Fig. 5). As shown in Figure 6a,b, TNF-α-stimulated HUVECs also showed increased NF-κB and AP-1 DNA-binding activities, whereas pretreatment of HUVECs with SalB for 18 h markedly attenuated TNF-α-stimulated NF-κB, and AP-1 activation in a dose-dependent manner.

DISCUSSION

SM has long been used in traditional Oriental herbal medicine for treatment of cardiovascular disorders [Zhu et al., 2001]. Previous studies have shown that an aqueous ethanolic extract of SM was effective in reducing endothelial damage and the severity of atherosclerosis [Wu et al., 1998; Chen et al., 2001b]. Treatment of human aortic vascular endothelial cells with SalB, a potent water-soluble antioxidant derived from SM, led to significant attenuation of TNF-α-induced ICAM-1 and VCAM-1 expression, suggesting that SalB may be responsible for the anti-atherosclerotic effects of SM [Chen et al., 2001a].

It is well accepted that endothelial dysfunction plays an important pathogenic role in diseases such as atherosclerosis, hypertension, diabetes, and thrombosis [Ross, 1999]. Dysregulation of the fibrinolytic potential of endothelial cells is actively involved in the development and progression of atherothrombosis. Increasing evidence suggests that proinflammatory cytokines play a major role in the development of atherosclerosis [Berk et al., 2001]. PAI-1, which plays a central role in regulating the fibrinolytic potential of endothelial cells, is produced by endothelial cells stimulated with various inflammatory cytokines, including TNF-α. Here, we studied the effect of SalB on PAI-1 expression and the accompanying signaling pathways in human endothelial cells. Importantly, we showed that SalB treatment lowered PAI-1 expression in TNF-α stimulated HUVECs. We further studied the action mechanisms of SalB by examining its effects on the ERK, JNK, and NF-κB pathways. We found that in addition to the NF-κB pathway, the JNK pathway seems to play a significant role in SalB-induced downregulation of PAI-1 expression.

TNF-α stimulates the release of PAI-1 from endothelial cells, thereby leading to accelerated coagulation. Both NF-κB-dependent and -independent intracellular signal transduction pathways are involved in TNF-α-induced PAI-1 expression, and TNF-α has been found to activate ERK, NF-κB, and JNK in HUVECs [Surapisitchat et al., 2001]. Here, in addition to the specific ERK (PD98059) and NF-κB (emodin) inhibitors previously reported [Hamaguchi et al., 2003], we used a JNK inhibitor (SP600125) to examine signaling during TNF-α-induced PAI-1 expression. We observed that PAI-1 expression was inhibited by all three tested inhibitors, and that SP600125, but not PD98059 or emodin, was additive to the inhibitory effects of SalB on TNF-α-induced PAI-1 secretion. These results indicate that the ERK and NF-κB pathway are involved in the SalB mediated inhibition of TNF-α-induced PAI-1 expression in HUVECs. The inhibitors show no significant effects on basal levels of PAI-1 production, which can be explained by the fact that the activities of ERK, JNK, and NF-κB

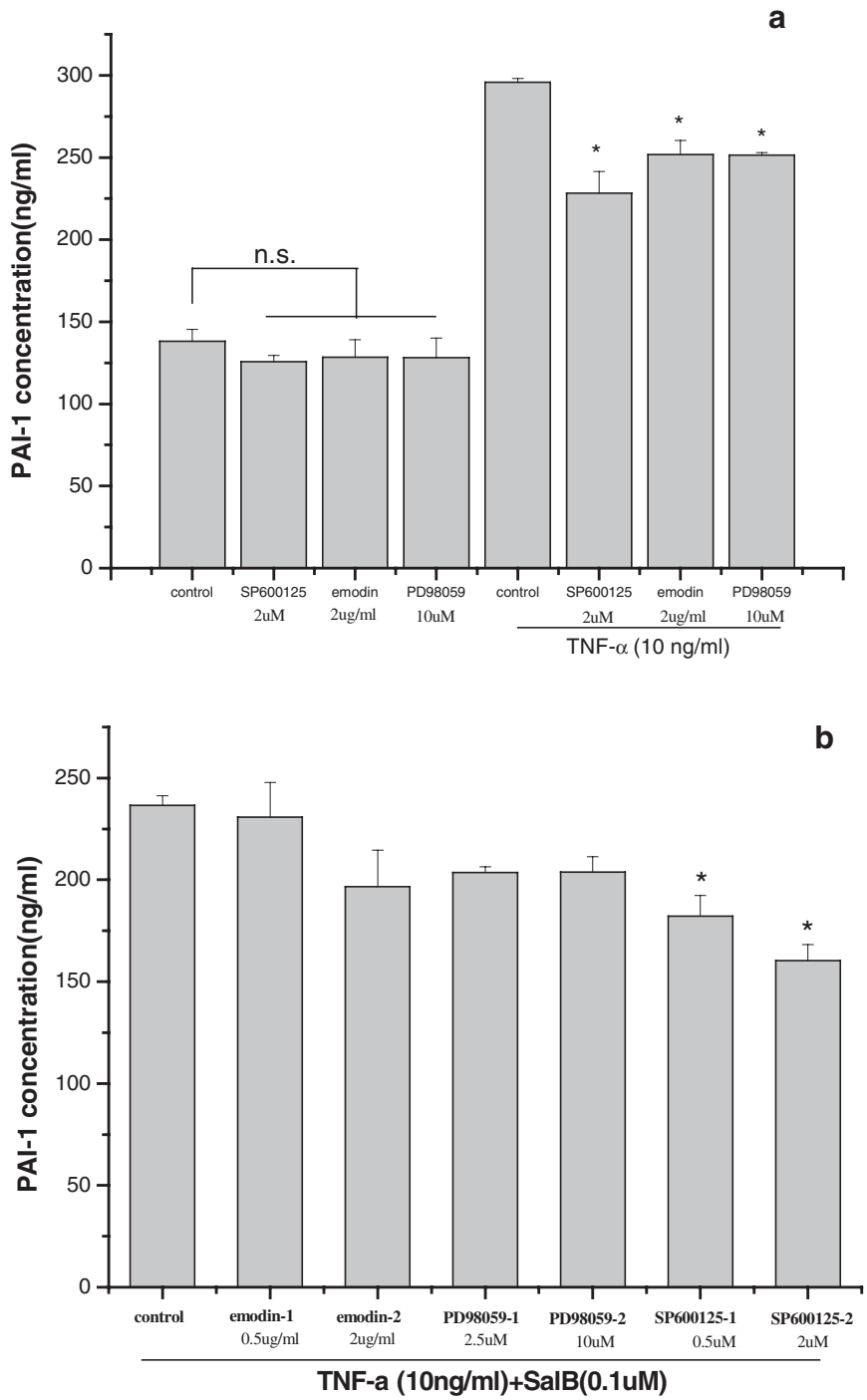

Fig. 4. Effect of transduction inhibitors on TNF-α-induced PAI-1 secretion in the presence (b) or absence (a) of SalB for 18 h. Values are expressed as mean ± SEM compared with control (*$P < 0.05$, n = 3).

are relatively low in the unstimulated cells [Berk et al., 2001; Yoshizumi et al., 2004].

As two major MAPK signaling pathways are reported to regulate AP-1 activity, one through ERK and the other through JNK [Karin, 1995], we analyzed the DNA binding activity of NF-κB and AP-1 in our cell system. Gel shift analyses revealed that NF-κB and AP-1 were activated in nuclear extracts of TNF-α-treated HUVECs (Fig. 6). The specificity of these bindings has been examined in detail by competition experiments performed with 100-fold excesses of

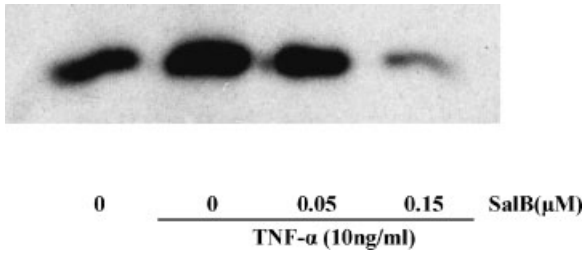

Fig. 5. Western blotting analysis of NF-κB p65 protein levels in cultured HUVEC nuclear extracts. Data shown are representative of three independent experiments.

unlabeled nucleotides corresponding to the NF-κB and AP-1 binding sequences (Fig. 6), and by Shift-Western blotting (data not shown) [Demczuk et al., 1993; Chen et al., 2003]. Elevated nuclear levels of p65 were observed in TNF-α treated HUVECs by Western blot analysis (Fig. 6), and SalB was shown to inhibit TNF-α-induced AP-1 activation, as well as activation and nuclear translocation of NF-κB (Fig. 6). Thus, the previous works together with our results seem to indicate that SalB decreases PAI-1 levels via inhibition of the ERK-AP-1 and NF-κB pathways.

The balance between PAI-1 and t-PA is known to determine hypofibrinolysis and procoagulant activity, and TNF-α seems to inhibit the net fibrinolytic system in HUVECs via production of PAI-I [Schleef et al., 1988]. There is some question as to whether TNF-α inhibits or stimulates t-PA production from endothelial cells. Schleef et al. [1988] reported an inhibitory effect in HUVECs, whereas Kawai et al. [1996] demonstrated that TNF-α stimulates t-PA production following shear stress in HUVECs. Hamaguchi et al. [2003] reported that TNF-α did not significantly affect t-PA production, and mentioned that t-PA release can differ between HUVEC lots. Thus, the effects of TNF-α on t-PA production and the fibrinolytic system remain to be clarified. When we investigated the effects of TNF-α and SalB on t-PA production, our results were consistent with those reported by Lopez et al. [2000], in which TNF-α modestly decreased t-PA production in HUVECs. Treatment with SalB did not significantly alter t-PA production. Thus, our results collectively indicate that SalB amplifies TNF-α-induced alterations in the fibrinolytic system by shifting the ratio of PAI-1 to t-PA. However, additional work will be required to elucidate whether SalB has beneficial effects on fibrinolytic systems in vivo.

Reliable data about the plasma SalB levels are scarce. After oral administration of the SM extract (the content of Sal B in SM extract was 28.1%) at the dose of 200 mg/kg body weight in Sprague–Dawley rats, the maximum plasm SalB concentration is 34 μM [Zhang et al., 2005]. Thus, oral ingestion of SM extract in rats would reach the levels of SalB used in the present studies. More work is needed, however, to determine the bioavailability and pharmacokinetics of SalB in human and experimental animal.

In conclusion, we herein showed that SalB inhibited TNF-α-induced PAI-1 mRNA expression and protein secretion, at least partially

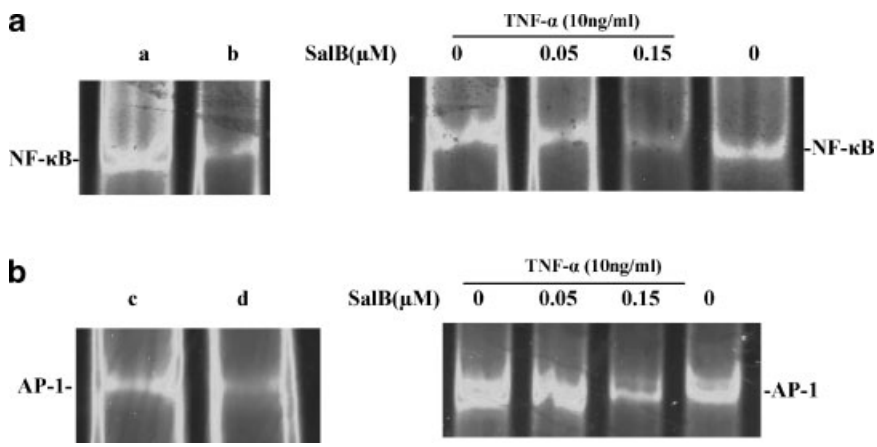

Fig. 6. False color image of NF-κB (a) and AP-1 (b) DNA binding activity measured by fEMSA. HUVECs were treated with or without TNF-α (10 ng/ml) in the presence of 0, 0.05, or 0.15 μM SalB for 18 h. Nuclear extracts were prepared and NF-κB and AP-1 DNA binding activities were measured by fEMSA. Competition experiments were performed with 100-fold excesses of unlabeled nucleotides corresponding to the NF-κB and AP-1 binding sequences; **Lane a**: 1 pM cy5 labeled NF-κB probe; (**Lane b**) 1 pM cy5 labeled NF-κB probe + 100 pM unlabeled NF-κB probe; (**Lane c**) 1 pM cy5 labeled AP-1 probe; (**Lane d**) 1 pM cy5 labeled AP-1 probe + 100 pM unlabeled AP-1 probe.

through inhibition of the ERK-AP-1- and NF-κB-dependent pathways. Our results will therefore add to previous works and may be helpful for the rational design of pharmacological strategies for treating or preventing atherosclerosis via regulation of PAI-1.

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