

# Salvianolic Acid B Attenuates VCAM-1 and ICAM-1 Expression in TNF- $\alpha$ -Treated Human Aortic Endothelial Cells

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**Abstract** Attachment to, and migration of leukocytes into the vessel wall is an early event in atherogenesis. Expression of cell adhesion molecules by the arterial endothelium may play a major role in atherosclerosis. It has been suggested that antioxidants inhibit the expression of adhesion molecules and may thus attenuate the processes leading to atherosclerosis. In the present study, the effects of a potent water-soluble antioxidant, salvianolic acid B (Sal B), and an aqueous ethanolic extract (SME), both derived from a Chinese herb, *Salvia miltiorrhiza*, on the expression of endothelial-leukocyte adhesion molecules by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-treated human aortic endothelial cells (HAECs) were investigated. When pretreated with SME (50 and 100  $\mu$ g/ml), the TNF- $\alpha$ -induced expression of vascular adhesion molecule-1 (VCAM-1) was notably attenuated (77.2 $\pm$ 3.2% and 80.0 $\pm$ 2.2%, respectively); and with Sal B (1, 2.5, 5, 10, and 20  $\mu$ g/ml), 84.5 $\pm$ 1.9%, 78.8 $\pm$ 1.2%, 58.9 $\pm$ 0.4%, 58.7 $\pm$ 0.9%, and 57.4 $\pm$ 0.3%, respectively. Dose-dependent lowering of expression of intercellular cell adhesion molecule-1 (ICAM-1) was also seen with SME or Sal B. In contrast, the expression of endothelial cell selectin (E-selectin) was not affected. SME (50  $\mu$ g/ml) or Sal B (5  $\mu$ g/ml) significantly reduced the binding of the human monocytic cell line, U937, to TNF- $\alpha$ -stimulated HAECs (45.7 $\pm$ 2.5% and 55.8 $\pm$ 1.2%, respectively). SME or Sal B significantly inhibited TNF- $\alpha$ -induced activation of nuclear factor kappa B (NF- $\kappa$ B) in HAECs (0.36- and 0.48-fold, respectively). These results demonstrate that SME and Sal B have anti-inflammatory properties and may explain their anti-atherosclerotic properties. This new mechanism of action of Sal B and SME, in addition to their previously reported inhibition of LDL, may help explain their efficacy in the treatment of atherosclerosis. *J. Cell. Biochem.* 82: 512–521, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** salvianolic acid B (Sal B); atherosclerosis; endothelial cell; VCAM-1; ICAM-1; E-selectin

The adhesion of circulating leukocytes to the vascular endothelium is a critical early event in the development of atherosclerosis [Joris et al., 1983; Faggitto et al., 1984]. This process

depends on the interaction between cell adhesion molecules expressed on the surface of endothelial cells (EC) and their cognate ligands on leukocytes [Price and Loscalzo, 1999]. Many adhesion molecules have been identified on ECs. These include vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and endothelial cell selectin (E-selectin) [Cybulsky and Gimbrone, 1991]. The increased expression of adhesion molecules by ECs in human atherosclerotic lesions may lead to further recruitment of leukocytes to atherosclerotic sites [van der Wal et al., 1992; O'Brien et al., 1993]. Although these inducible molecules have received con-

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siderable attention, little is known about the effects of antioxidants on adhesion molecules, and a better understanding of the mechanisms of action of antioxidants in terms of cell adhesion molecule expression may provide important insights into the prevention of atherogenesis.

*Salvia miltiorrhiza* Bunge (SM) is a herb often used in popular folk medicine in China, Japan, and Taiwan for the treatment of cardiovascular disorders (called blood stasis in traditional Chinese medicine) [Lei and Chiou, 1986]. Treatment with SM reduces the intimal thickness in the air-injured carotid artery in rats and inhibits the proliferation of isolated rabbit arterial smooth muscle cells (SMCs) [Zhou et al., 1996]. Salvianolic acid B (Sal B), a water-soluble polyphenolic antioxidant isolated from the roots of the same plant, scavenges 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and inhibits LDL oxidation more effectively than the antioxidant, probucol [Wu et al., 1998]. A Sal B-rich fraction of a SM extract (SME) also inhibits LDL oxidation in vitro and ex vivo and reduces atherosclerosis in hypercholesterolemic rabbits [Wu et al., 1998].

The possible reduction by antioxidants of adhesion molecule expression could play a key role in the prevention or treatment of cardiovascular disorders. We therefore tested the ability of SME and Sal B to modulate the expression of adhesion molecules and transcriptional factor NF- $\kappa$ B by human aortic endothelial cells (HAECs). Our study demonstrated that SME or Sal B attenuated the expression of VCAM-1 and ICAM-1 in a dose-dependent manner, and that this effect was mediated by partial blockage of NF- $\kappa$ B expression. Both agents also significantly inhibited adhesion of the human monocytic cell line, U937, to HAECs.

## MATERIALS AND METHODS

### Reagents

Probuco1 [(4,4'-(isopropylenedithio)bis(2,6-di-butyl-phenol)], was obtained from Sigma and dissolved in ethanol as a 10 mM stock solution. Dry roots of SM were extracted for 24 h at room temperature with a 4:1 (vol/vol) mixture of water and ethanol. After evaporation of the solvent under reduced pressure, this extract (SME) was stored under nitrogen at 4°C until use. The concentration of Sal B in the SME was 4.4% by weight as determined by reversed-

phase HPLC. Since Sal B is water-soluble and has low pKa values similar to that of a carboxylic acid, the content of Sal B was further enriched by adjustment of the pH of the concentrated SME and precipitation with acetone-water. After ion exchange and Sephadex LH-20 column chromatography, Sal B was obtained at greater than 98% purity. For use, it was dissolved as a 14 mM stock solution in PBS. Unless specified otherwise, all reagents were from Sigma (Missouri).

### Culture of HAECs

HAECs were provided as cryopreserved tertiary cultures by Cascade Biologics (Oregon) and were grown in culture flasks in endothelial cell growth medium (Cascade Biologics) supplemented with fetal bovine serum (FBS, 2%), human epidermal growth factor (10 ng/ml), human fibroblast growth factor (3 ng/ml), heparin (10  $\mu$ g/ml), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and Fungizone (1.25  $\mu$ g/ml). The growth medium was changed every other day until confluence. Cells under passage 8 were used for this study. The purity of HAEC cultures was verified by staining with monoclonal antibody against von-Willebrand factor.

### Measurement of Cytotoxicity

Mitochondrial dehydrogenase activity was measured as an index of cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Welder [1992]. In brief, MTT (0.5 mg/ml) was applied to cells for 4 h to allow the conversion of MTT into formazan crystals, then, after washing with phosphate-buffered saline (PBS), the cells were lysed with dimethyl sulfoxide (DMSO), and the absorbance read at 530 and 690 nm with a DIAS Microplate Reader (Dynex Technologies, Virginia). The reduction in optical density (OD) caused by cytokine and drug treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

### Enzyme-Linked Immunosorbent Assay (ELISA)

To measure the cell-surface expression of adhesion molecules, HAECs in 96-well plates were pretreated with probucol, SME, or Sal B at the indicated concentrations for 18 h, then stimulated for 6 h at 37°C with 2 ng/ml TNF- $\alpha$ . Expression of cell-surface VCAM-1, ICAM-1,

and E-selectin was measured by separate incubation for 30 min at room temperature with specific goat antibodies against human VCAM-1, ICAM-1, or E-selectin (0.5  $\mu\text{g}/\text{ml}$ , Research and Development), then with horseradish peroxidase-conjugated rabbit anti-goat IgG (0.5  $\mu\text{g}/\text{ml}$ ) for 1 h at room temperature, the cells being washed with HBSS containing 2% skim milk after each incubation step. Binding of the secondary antibody was determined by incubating the plates in the dark for 15 min with 100  $\mu\text{l}$  of 3% *o*-phenylenediamine and 0.03%  $\text{H}_2\text{O}_2$  in 50 mM citrate buffer and 100 mM phosphate buffer, then terminating the reaction by addition of 50  $\mu\text{l}$  of 2 M  $\text{H}_2\text{SO}_4$ . Surface expression of adhesion molecules was quantified by reading the OD at 490 nm in an ELISA plate reader.

#### Western Blots

For Western blotting, the cells were washed with PBS, centrifuged at 1,200g for 10 min at 4°C, then lysed for 1 h at 4°C with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05 % SDS, 0.5 % Triton X-100, and 1 mM PMSF) and centrifuged at 4,000g for 30 min at 4°C. The supernatants were applied to 8% SDS-PAGE and transferred at room temperature by blotting to polyvinylidene difluoride (PVDF) membrane (NEN), which were then treated for 1 h at room temperature with PBS-Tween 20 (0.05%)/2% skim milk and separately incubated for 1 h at room temperature with goat anti-human VCAM-1, ICAM-1, and E-selectin antibodies. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat monoclonal antibodies. Antigen detection was performed via Chemiluminescence Reagent Plus (NEN) and exposure to Biomax MR film (Kodak).

#### Adhesion Assay

HAECs ( $5 \times 10^5$ ) were distributed into 24-well plates before the assay and were allowed to reach confluence, then the growth medium was supplemented with probucol, SME, or Sal B at the indicated concentrations for 18 h, followed by 2 ng/ml TNF- $\alpha$  for 6 h at 37°C. U937 cells, originally derived from a human histiocytic lymphoma, were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium (M.A. Bioproducts, Walkersville, MD) containing 5% FBS, and subcultured at a 1:5 ratio three times

per week. They were labeled for 1 h at 37°C with 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM, 10  $\mu\text{M}$ , Boehringer-Mannheim) in serum-free RPMI 1640 media, then washed with PBS to remove free dye and resuspended in RPMI 1640 containing 2% FBS.  $10^6$  labeled U937 cells were added to each HAEC-containing well and incubation continued for 1 h. Non-adherent cells were removed by two gentle washes with PBS, then the number of bound U937 cells was determined by counting four different fields using a fluorescence microscope and an ocular grid and a 20 $\times$  objective. Fields for counting adherent cells were randomly located at a half-radius distance from the center of the monolayers.

#### NF- $\kappa$ B p65 Expression

To measure NF- $\kappa$ B expression, confluent HAECs on slides were pretreated with or without test compounds for 18 h, then exposed to TNF- $\alpha$  (2 ng/ml) for 15 min. They were then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 min at 4°C, washed with PBS, blocked with 5% BSA in PBS for 1 h at room temperature, then reacted for 1 h at room temperature with mouse anti-human NF- $\kappa$ B p65 antibody (1: 500 dilution in PBS; Transduction). After washes, the slides were incubated for 1 h at 37°C with FITC-conjugated goat anti-mouse IgG, then viewed on a fluorescent microscope. Nuclear extracts containing NF- $\kappa$ B protein were prepared according to the method of Ji et al. [1998]. In brief, the cells were washed with cold PBS, then scraped off the plates in 0.6 ml of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, 1.0 mM PMSF, and 1.5 mM  $\text{MgCl}_2$ ). After centrifugation at 300g for 10 min at 4°C, they were resuspended in 80  $\mu\text{l}$  of buffer B (0.1% Triton X-100 in buffer A), left on ice for 10 min, then centrifuged at 12,000g for 10 min at 4°C. The nuclear pellets were resuspended in 70  $\mu\text{l}$  of ice-cold buffer C (20 mM HEPES, pH 7.9, 1.5 mM  $\text{MgCl}_2$ , 0.42 M NaCl, 1 mM DTT, 0.2 mM EDTA, 1.0 mM PMSF, and 25% glycerol), shaken for 30 min at 4°C, then centrifuged at 15,000g for 30 min at 4°C. A fixed amount of protein was separated on a 10% SDS-PAGE and transferred to PVDF membranes, which were then incubated with primary mouse anti-human NF- $\kappa$ B p65 antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG (0.5  $\mu\text{g}/\text{ml}$ ) for 1 h at 37°C. Immunoreactivity was

detected via Chemiluminescence Reagent Plus (NEN) and exposed to Biomax MR film (Kodak).

### Statistical Analysis

Results were expressed as the mean $\pm$ SEM. Data were analyzed by ANOVA followed by the Dunnett's test. A *P* value less than 0.05 was considered statistically significant.

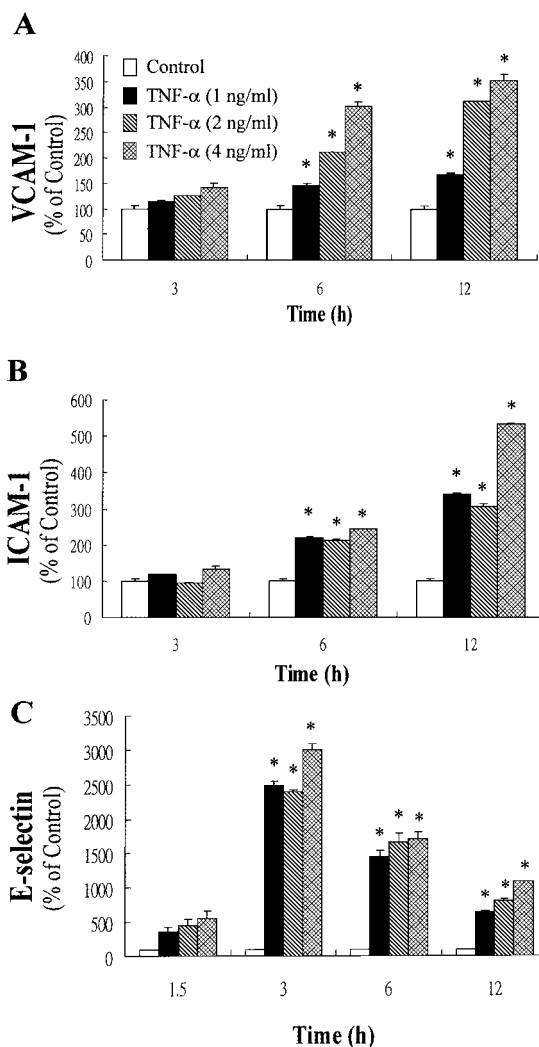
## RESULTS

### TNF- $\alpha$ Induces Adhesion Molecule Expression on HAECs in a Time- and Dose-Dependent Manner

To determine the optimal conditions for TNF- $\alpha$ -induced adhesion molecule expression by HAECs, we first performed dose-response studies in which HAECs were cultured with various concentrations of TNF- $\alpha$  for various time intervals. As shown in Figure 1A, VCAM-1 expression was induced in a dose-dependent manner after treatment with 1, 2, or 4 ng/ml of TNF- $\alpha$  for 6 h (145.0 $\pm$ 3.4%, 211.6 $\pm$ 1.5%, or 302.0 $\pm$ 7.0%, respectively, of control levels) or 12 h (168.0 $\pm$ 2.0%, 312.0 $\pm$ 0.8%, or 352.0 $\pm$ 12.0%, respectively), but not after 3 h of treatment (115.2 $\pm$ 1.6%, 125.9 $\pm$ 2.0%, or 140.8 $\pm$ 8.8%, respectively). A similar time-course was seen for ICAM-1 expression (116.7 $\pm$ 0.7%, 94.2 $\pm$ 1.0%, or 131.9 $\pm$ 8.2% after 3 h; 218.1 $\pm$ 3.6%, 211.6 $\pm$ 1.9%, or 242.0 $\pm$ 2.9% after 6 h; 339.9 $\pm$ 2.2%, 305.1 $\pm$ 7.0%, or 531.9 $\pm$ 4.3% after 12 h), but no dose-dependency was seen over the range tested (Fig. 1B). E-selectin expression, which again showed no dose-dependency, reached a maximum after 3 h (2,487.5 $\pm$ 57.8%, 2,383.7 $\pm$ 29.5%, or 2,991.3 $\pm$ 100.1%, respectively), then declined (Fig. 1C). Treatment with 2 ng/ml of TNF- $\alpha$  for 6 h was found to significantly up-regulate the expression of VCAM-1, ICAM-1, and E-selectin and these conditions were therefore used throughout the study, unless otherwise stated.

### Toxicity of Antioxidants for HAECs

Cell viability was assessed by Trypan blue exclusion and the MTT assay. Treatment of HAECs with 1, 2, or 4 ng/ml of TNF- $\alpha$  did not result in cytotoxicity (data not shown). In the presence of 2.5, 5, or 10  $\mu$ M probucol, cell viability was higher than 98%. When incubated with 25, 50, 100, or 200  $\mu$ g/ml of SME, cell viability was 115.4 $\pm$ 3.2%, 110.3 $\pm$ 2.2%, 100.6 $\pm$ 0.2%, or 71.6 $\pm$ 0.8%, respectively, 200  $\mu$ g/ml SME causing a significant

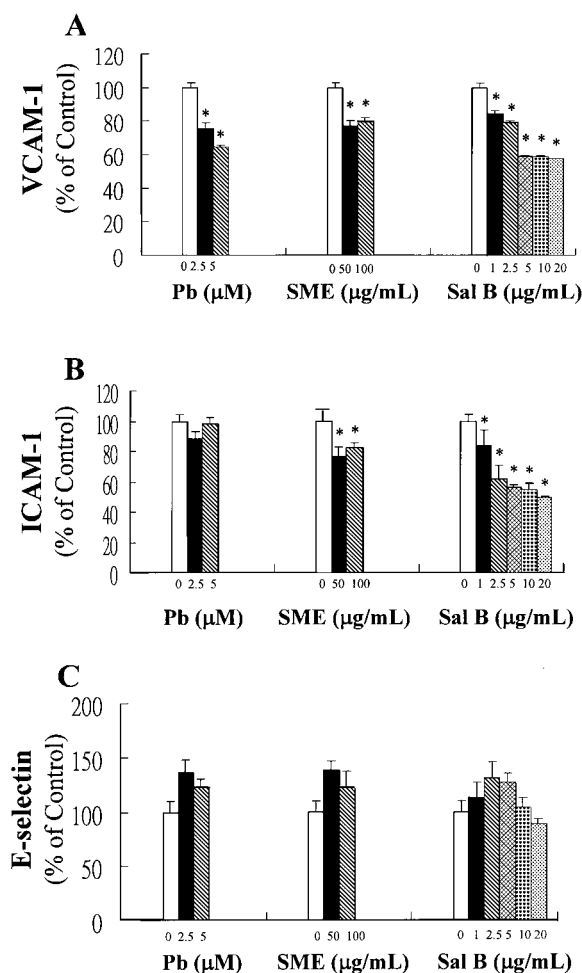


**Fig. 1.** Dose- and time-dependent responses of VCAM-1 (A), ICAM-1 (B), and E-selectin (C) expression induced by TNF- $\alpha$  as assessed by ELISA. Data are expressed as the mean $\pm$ SEM of three separate experiments performed in triplicate. The percentage of adhesion molecule expression induced by different concentration of TNF $\alpha$  was compared to that in controls (\**P* < 0.05).

reduction in cell viability. Treatment with 2.5, 5, 10, or 20  $\mu$ g/ml of Sal B did not affect cell viability (102.2 $\pm$ 1.3%, 98.6 $\pm$ 0.4%, 104.1 $\pm$ 2.0%, or 97.9 $\pm$ 2.1%, respectively).

### Sal B and SME Decrease the Expression of VCAM-1 and ICAM-1, but not E-Selectin, by TNF- $\alpha$ -Stimulated HAECs

The effects of antioxidants on the expression of VCAM-1, ICAM-1, and E-selectin by HAECs were determined. HAECs were pretreated for 18 h with probucol, SME, or Sal B before addition of 2 ng/ml TNF- $\alpha$ . Probucol (2.5 or



**Fig. 2.** Effect of probucol, SME, or Sal B on adhesion molecule expression in HAECs. Cells were treated for 18 h with probucol, SME, or Sal B prior to the addition of TNF $\alpha$  (2 ng/ml). Expression of adhesion molecules VCAM-1 (A), ICAM-1 (B), and E-selectin (C) was detected by ELISA. Data are expressed as the mean  $\pm$  SEM of three experiments performed in triplicate. \*indicates  $P < 0.05$  compared with TNF- $\alpha$ -treated HAECs (controls).

5  $\mu$ M) caused a significant dose-dependent decrease in VCAM-1 expression (75.1  $\pm$  4.0% and 64.3  $\pm$  1.5% expression, respectively, compared to TNF- $\alpha$ -treated HAECs) (Fig. 2A), but had no significant effect on either ICAM-1 or E-selectin expression (Fig. 2B and C). SME treatment (50 or 100  $\mu$ g/ml) caused significant down-regulation of expression of VCAM-1 (77.2  $\pm$  3.2% and 80.0  $\pm$  2.2% expression, respectively) and ICAM-1 (76.0  $\pm$  2.3% and 81.6  $\pm$  1.5% expression, respectively) (Fig. 2A and B). Sal B (1, 2.5, 5, 10, or 20  $\mu$ g/ml) treatment inhibited TNF- $\alpha$ -induced expression of VCAM-1 (84.5  $\pm$  1.9%, 78.8  $\pm$  1.2%, 58.9  $\pm$  0.4%, 58.7  $\pm$  0.9%, or 57.4  $\pm$  0.3% expression, respectively) and

ICAM-1 (83.8  $\pm$  3.3%, 61.3  $\pm$  3.1%, 55.5  $\pm$  0.8%, 53.9  $\pm$  1.5%, or 49.3  $\pm$  0.2% expression, respectively). Neither SME nor Sal B had any significant effect on E-selectin expression (Fig. 2C).

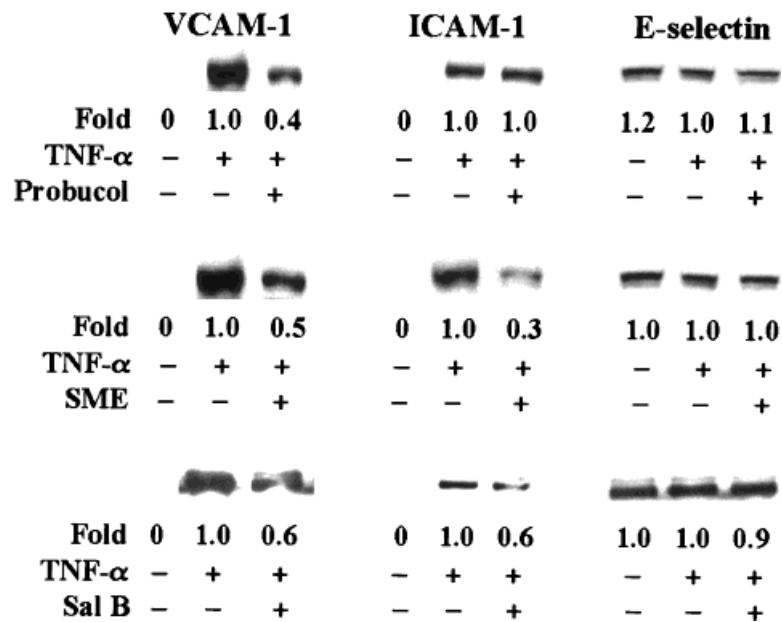
To confirm these findings, Western blot analysis was performed (Fig. 3). Amounts of VCAM-1 and ICAM-1 were very low in control untreated HAECs, but their expression increased after TNF- $\alpha$  stimulation. Interestingly, E-selectin was constitutively expressed in HAECs. Pretreatment with probucol (5  $\mu$ M), SME (50  $\mu$ g/ml), or Sal B (5  $\mu$ g/ml) significantly inhibited VCAM-1 expression, and SME or Sal B pretreatment also resulted in reduced ICAM-1 expression. None of the three treatments, including TNF- $\alpha$  alone, had any effect on E-selectin expression.

#### Sal B and SME Inhibit Binding of U937 Cells to TNF- $\alpha$ -Stimulated HAECs

The effects of antioxidants on the binding of U937 cells to TNF- $\alpha$ -stimulated HAECs were determined (Fig. 4). Control confluent HAECs showed minimal binding to U937 cells (data not shown), but adhesion was substantially increased when the HAECs were treated with TNF- $\alpha$ . Pretreatment with probucol (5  $\mu$ M), SME (50  $\mu$ g/ml), or Sal B (5  $\mu$ g/ml) significantly reduced the adhesion of U937 cells to TNF- $\alpha$ -stimulated HAECs (47.7  $\pm$  9.2%, 45.7  $\pm$  2.5%, or 55.8  $\pm$  1.2% inhibition, respectively).

#### Sal B and SME Attenuate NF- $\kappa$ B p65 Expression in TNF- $\alpha$ -Stimulated HAECs

To determine whether NF- $\kappa$ B activation was involved in the pretranslational effects of antioxidants on adhesion molecule expression, we studied NF- $\kappa$ B p65 protein levels in the nuclei of TNF- $\alpha$ -treated HAECs by immunofluorescence and Western blots. TNF- $\alpha$ -stimulated HAECs showed marked NF- $\kappa$ B p65 staining in the nuclei, while probucol-, SME-, or Sal B-pretreated cells showed weaker NF- $\kappa$ B expression in the nuclei, but stronger staining in the cytoplasm (Fig. 5A). Consistent with the in situ findings, when analyzed by Western blots, higher levels of NF- $\kappa$ B p65 protein were found in the nuclei of TNF- $\alpha$ -stimulated HAECs compared with control HAECs. Furthermore, probucol (5  $\mu$ M), SME (50  $\mu$ g/ml), or Sal B (5  $\mu$ g/ml) pretreatment significantly reduced NF- $\kappa$ B expression (52%, 36%, or 48% expression compared to TNF- $\alpha$ -treated cells, respectively) (Fig. 5B).



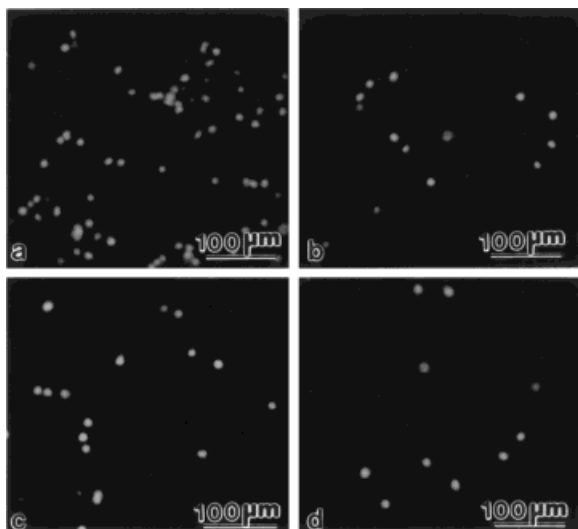
**Fig. 3.** Western blot analysis of VCAM-1, ICAM-1, and E-selectin protein levels in cultured HAECs. Cells were stimulated for 18 h with probucol (5  $\mu$ M), SME (50  $\mu$ g/ml), or Sal B (5  $\mu$ g/ml) prior to the addition of TNF $\alpha$  (2 ng/ml). Three independent experiments gave similar results.

## DISCUSSION

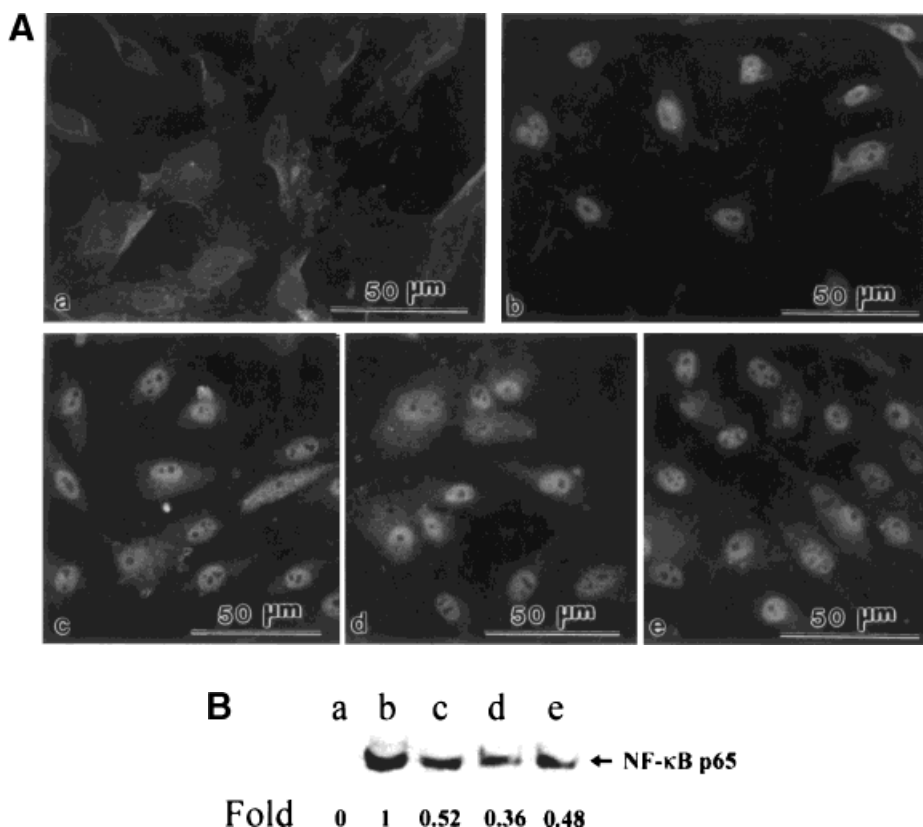
In the present report, we found that TNF- $\alpha$  treatment of cultured HAECs induced VCAM-1, ICAM-1, and E-selectin expression in a time-

dependent and, in the case of VCAM-1, in a dose-dependent, manner. Sal B and SME treatment effectively blocked VCAM-1 and ICAM-1 expression in TNF- $\alpha$ -stimulated HAECs, while probucol only inhibited VCAM-1 expression, none of the treatments affected E-selectin expression. In addition, all three treatments inhibited the binding of the human monocytic cell line, U937, to TNF- $\alpha$ -stimulated HAECs.

We tested the effects of SME, as it is derived from a Chinese herb, SM. Nonpolar extracts of the plant contain tanshinones, which inhibit platelet aggregation [Onitsuka et al., 1983] and protect the myocardium against ischemia-induced derangement [Yagi et al., 1989]. SME, an aqueous ethanolic extract of SM, is rich in polyphenolic compounds that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage [Liu et al., 1992], one of these, Sal B, is a potent hepatoprotective agent and water-soluble antioxidant. SME scavenges DPPH radicals and inhibits Cu<sup>2+</sup>-induced LDL oxidation [Wu et al., 1998]. New Zealand white rabbits on a high cholesterol diet show reduced endothelial damage and severity of atherosclerosis when treated with SME [Wu et al., 1998]. LDLs from SME-treated animals contain more vitamin E and are more resistant to oxidation *ex vivo* [Wu et al., 1998]. In this study, we found that



**Fig. 4.** Effects of probucol, SME, or Sal B on the adhesion of U937 cells to TNF- $\alpha$ -stimulated HAECs. Cells were pretreated for 18 h with probucol (5  $\mu$ M), SME (50  $\mu$ g/ml), or Sal B (5  $\mu$ g/ml), followed by TNF- $\alpha$ . Representative fluorescent photomicrographs show effects of drug treatment on the TNF- $\alpha$ -induced adhesion of fluorescein-labeled U937 cells to HAECs. **a:** TNF- $\alpha$  treatment; **b:** probucol + TNF- $\alpha$  treatment; **c:** SME + TNF- $\alpha$  treatment; **d:** Sal B + TNF- $\alpha$  treatment.



**Fig. 5.** NF- $\kappa$ B expression by TNF- $\alpha$ -stimulated HAECs pretreated with probucol, SME, or Sal B. **A:** Immunofluorescent staining of NF- $\kappa$ B p65. a: normal HAECs; b: TNF- $\alpha$  treatment; c: probucol + TNF- $\alpha$  treatment; d: SME + TNF- $\alpha$  treatment; e: Sal B + TNF- $\alpha$  treatment. **B:** Western blotting and densitometry of NF- $\kappa$ B in nuclear extracts of HAECs. The results are representative of three separate experiments.

pretreatment with SME or Sal B significantly attenuated TNF- $\alpha$ -induced VCAM-1 and ICAM-1 expression. These findings are consistent with the hypothesis that endothelial dysfunction, such as disturbances in adhesion molecule expression, plays an important role in the pathogenesis of atherosclerosis.

Recruitment of circulating mononuclear cells and endothelial-mononuclear cell adhesion are crucial in the development of atherosclerosis. The process by which leukocytes become adherent to the endothelium is the result of complex choreography requiring the sequential, yet overlapping, functions of many classes of adhesion molecules [Munro, 1993; Price and Loscalzo, 1999]. Tethering and rolling, the first steps in recruitment, appear to depend on the interaction of P- and E-selectin with carbohydrate ligands on leukocytes. Firm adhesion follows if the leukocytes encounter activating signals while rolling along the endothelium, and is facilitated by the interaction of very late antigen-4 (VLA-4) with VCAM-1 or of lympho-

cyte function antigen-1 (LFA-1) with ICAM-1 [Marlin and Springer, 1987; Elices et al., 1990]. Diapedesis and transmigration are dependent on a chemotactic gradient, ICAM-1 activation, and platelet/endothelial cell adhesion molecule (PECAM-1), located at the intercellular junction. Consistent with the results of Marui et al. [1987], the present study showed that TNF- $\alpha$ -treated endothelial cells show significant expression of VCAM-1, ICAM-1, and E-selectin. E-selectin is expressed at an earlier stage, peaks at about 3 h, then progressively disappears. A similar trend was observed by Bevilacqua et al. [1987], who showed that E-selectin expression occurs relatively early following agonist stimulation, peaks at about 4 h, and is greatly reduced by 24 h. Enhancement of VCAM-1 and ICAM-1 expression developed more gradually than E-selectin expression. Enhancement of basal ICAM-1 expression has previously been reported to develop more gradually than E-selectin expression and to plateau at ~24 h [Dustin et al., 1986].

This study also demonstrates that TNF- $\alpha$ -induced VCAM-1 expression is significantly reduced in HAECs pretreated with probucol, whereas ICAM-1 and E-selectin expression was less affected. Cominacini et al. [1997] have previously reported that pretreatment of human umbilical vein endothelial cells (HUVECs) with 5  $\mu$ M probucol for 18 h significantly reduces the expression of VCAM-1 and ICAM-1 induced by oxidized LDL (oxLDL), this effect was significantly greater for VCAM-1 than for ICAM-1. Probucol also substantially abrogates LPS-induced E-selectin expression in HUVECs, whereas ICAM-1 expression is not affected [Kaneko et al., 1996]. The difference between these findings may be related to differences in cell types (HAECs and HUVECs) and the cytokines and inducers (TNF- $\alpha$ , oxLDL, and LPS). This is the first report using HAECs as a model to study the effect of Sal B on the expression of cell adhesion molecules.

In contrast, Sal B and SME pretreatment did not inhibit the TNF- $\alpha$ -induced expression of E-selectin. One plausible explanation for the different effects on the expression of E-selectin, VCAM-1, and ICAM-1 is that they share common regulatory signals immediately after receptor activation by TNF- $\alpha$ , but the expression of these molecules may be modulated by gene-specific signal transduction mechanisms. The reason why E-selectin gene expression escapes inhibition by antioxidants is unknown.

The Sal B- and SME-induced decrease in monocyte-EC adhesion has important implications in terms of atherogenic mechanisms, as well as in the treatment of atherosclerosis. SME has been shown to reduce endothelial damage, cholesterol deposition, and atherosclerotic areas in aortas of cholesterol-fed rabbits [Wu et al., 1998]. Atherosclerotic lesions result from the accumulation of foam cells of monocyte/macrophage origin within the arterial intima [Joris et al., 1983; Faggitto et al., 1984]. Based on the probable involvement of VCAM-1 and ICAM-1 in monocyte recruitment to early atherosclerotic lesions, our findings suggest an additional mechanism by which Sal B and SME may be involved in preventing the progress of atherosclerosis.

The translocation of the transcription factor, NF- $\kappa$ B, is involved in the signal transduction pathways for TNF- $\alpha$ -induced adhesion molecule expression [Lenardo and Baltimore, 1989]. NF- $\kappa$ B is expressed as a multiunit transcription

factor that can be activated by diverse signals, possibly through phosphorylation of the I $\kappa$ B subunit. Its dissociation from the inactive cytoplasmic complex is followed by translocation of the active p50/p65 dimer to the nucleus [Ghosh and Baltimore, 1990]. In this study, TNF- $\alpha$ -treated HAECs were found to contain elevated levels of nuclear NF- $\kappa$ B p65. Marui et al. [1993] have reported that expression of VCAM-1, ICAM-1, E-selectin, and NF- $\kappa$ B is upregulated in TNF- $\alpha$ -stimulated HUVECs. Antioxidants, such as N-acetylcysteine (NAC) and other cysteine derivatives, inhibit the NF- $\kappa$ B-driven transcription of HIV-1 and HIV-1 viral replication [Mihm et al., 1991]. In several immortalized cell lines, NF- $\kappa$ B is activated by diverse stimuli, such as TNF- $\alpha$ , IL-1 $\beta$ , LPA, and PIC, and inhibited by the antioxidants, pyrrolidine dithiocarbamate and NAC [Schreck et al., 1992]. Our study demonstrates a similar pattern of antioxidant (SME and probucol)-sensitive inactivation of VCAM-1 expression and NF- $\kappa$ B-like activity in HAECs.

Our study supports the notion that NF- $\kappa$ B-like factors are required for activation of VCAM-1 expression in endothelial cells. However, an important question is raised about the role of NF- $\kappa$ B in E-selectin and ICAM-1 gene expression. Based on DNA transfection analysis of deleted promoter constructs of the E-selectin gene, a cis-acting promoter element, which contains a NF- $\kappa$ B consensus binding site, has been found to be required to mediate IL-1 $\beta$  transcriptional activity [Ghersa et al., 1992]. However, we found that SME or probucol did not inhibit E-selectin induction. SME or probucol inhibition of NF- $\kappa$ B-like activation, but not of E-selectin expression, argues against NF- $\kappa$ B-like transcriptional factors being essential components in E-selectin gene activation. Similarly, these inducible factors may not be essential for the activation of ICAM-1 expression, despite the presence of NF- $\kappa$ B consensus DNA binding sites on the ICAM-1 promoter [Degitz et al., 1991]. These results suggest that other components of the transcription factor complex, such as activator protein-1, may be involved in regulating the expression of adhesion molecules in response to the redox status in HAECs [Sen and Packer, 1996].

In conclusion, this study shows that TNF- $\alpha$  induces the expression of VCAM-1, ICAM-1, and E-selectin in HAECs. This is the first study to show that SME, which contains Sal B as a



predominant, water-soluble antioxidant, reduces the expression of adhesion molecules and consequently decreases leukocyte adhesion to HAECs. Since monocyte recruitment into the vascular wall after their adhesion to endothelial cells is a crucial step in the pathogenesis of atherosclerosis, our study implies that antioxidants may have as yet unexplored therapeutic potential in the prevention of atherosclerosis. In doing so, these water-soluble polyphenolic antioxidants, such as Sal B, may have an additional beneficial effect in multiple pathological events involving leukocyte adhesion, including inflammation and atherosclerosis.

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