# Hypoxia-Induced Mitogenic Factor Promotes Vascular Adhesion Molecule-1 Expression via the PI-3K/Akt–NF-κB Signaling Pathway

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**Hypoxia-induced mitogenic factor (HIMF), also known as FIZZ1 (found in inflammatory zone 1), is an important player in lung inflammation. However, the effects of HIMF on cell adhesion molecules involved in lung inflammation remain largely unknown. In the present work, we tested whether HIMF modulates vascular adhesion molecule (VCAM)-1 expression, and dissected the possible signaling pathways that link HIMF to VCAM-1 upregulation. Recombinant HIMF protein, instilled intratracheally into adult mouse lungs, results in a significant increase of VCAM-1 production in vascular endothelial, alveolar type II, and airway epithelial cells. In cultured mouse endothelial SVEC 4-10 and lung epithelial MLE-12 cells, we demonstrated that HIMF induces VCAM-1 expression via the phosphatidylinositol-3 kinase (PI-3K)/Akt–nuclear factor (NF)-κB signaling pathway. Knockdown of HIMF expression by small interference RNA attenuated LPS-induced VCAM-1 expression *in vitro*. We showed that HIMF induced phosphorylation of the IκB kinase signalsome and, subsequently, IκBα, leading to activation of NF-κB. Meanwhile, VCAM-1 production was correspondingly upregulated. Blocking NF-κB signaling pathway by expression of dominant-negative mutants of IκB kinase and IκBα suppressed HIMF-induced VCAM-1 upregulation. HIMF also strongly induced phosphorylation of Akt. A dominant-negative mutant of PI-3K, Δp85, as well as PI-3K inhibitor, LY294002, also blocked HIMF-induced NF-κB activation and attenuated VCAM-1 production. Furthermore, LY294002 pretreatment abolished HIMF-enhanced mononuclear cells adhesion to endothelial and epithelial cells. Our findings connect HIMF to signaling pathways that regulate inflammation, and thus reveal the critical roles that HIMF plays in lung inflammation.**

**Keywords:** gene expression; hypoxia-induced mitogenic factor; signal transduction; vascular adhesion molecule-1

Leukocyte migration into the alveolar compartments is a prominent feature of acute and chronic lung inflammation (1), and the process involves several steps. The initial interaction between circulating leukocytes and endothelium appears to be transient, resulting in the rolling of leukocytes along the vessel wall (2). The rolling leukocytes then become activated by local factors generated by the endothelium, resulting in their arrest and firm adhesion to the vessel wall. Finally, the leukocyte transmigrates through the endothelium (2). These complex processes are regulated, in part, by specific endothelial-leukocyte adhesion molecules (3). One of primary importance is vascular adhesion molecule (VCAM)-1, a member of the immunoglobulin gene superfamily that mediates leukocyte binding to the endothelial cell through its interaction with the leukotyte's integrin counter-receptor very late activation antigen (VLA)-4 (4). Because of the selective expression of VLA-4 on monocytes and lymphocytes, but not neutrophils, VCAM-1 plays an important role in mediating mononuclear leukocyte-selective adhesion (5).

Cytokines commonly found in inflammatory atherogenic lesions, such as TNF-α and IL-1, induce the concurrent expression of VCAM-1, intercellular adhesion molecule (ICAM)-1, and E-selectin in cultured endothelial cells (6). The elevated and prolonged expression of VCAM-1 has been observed in both experimental models and human inflammatory processes. Treatment of endothelial cells with bacterial LPS *in vitro* as well as administration of endotoxin *in vivo* upregulates VCAM-1 expression, especially in lung and liver (7, 8). Recent reports indicate that, during the lung inflammation process, alveolar epithelial cells are likely important not only for retention and activation of leukocytes, but also for regulating their passage into the airway (9). VCAM-1 upregulation via protein kinase C δ–p38 kinase–linked cascade mediated the TNF-α–induced elevation of leukocyte adhesion and migration in the airway epithelium (9). Given the functional and clinical implications of these findings, efforts have been made to better understand the mechanism modulating VCAM-1 expression in endothelial and epithelial cells.

Previously, from a mouse model of hypoxia-induced pulmonary hypertension, we discovered a highly upregulated gene named hypoxia-induced mitogenic factor (HIMF) (10). HIMF shares homologies with FIZZ1 (found in inflammatory zone 1), a protein identified in a mouse lung inflammation model (11), and with resistin-like molecule-α in adipose tissue (12). Holcomb and colleagues first reported FIZZ1 as an abundantly secreted protein in the bronchoalveolar lavage fluid of a murine asthmatic model (11). They observed the secretion of FIZZ1 from the inflamed airway epithelium and type 2 pneumocytes, and demonstrated that FIZZ1 could inhibit the action of nerve growth factor *in vitro* (11). During allergic pulmonary inflammation induced by ovalbumin challenge, FIZZ1 expression markedly increases in hypertrophic and hyperplasic bronchial epithelium, and also appears in alveolar type 2 cells (11). More recently, FIZZ1 has been implicated in mediating the deposition of extracellular matrix in an animal model of lung fibrosis (13). These studies suggest that FIZZ1 plays an important role in lung inflammation.

In the present study, we tested the hypothesis that HIMF is functioning in a cytokine-like manner, modulating VCAM-1 production. We investigated the molecular mechanisms of VCAM-1 upregulation mediated by HIMF in mouse lungs, cultured endothelial and lung epithelial cells, and dissected the

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possible signaling pathways. Our results connect HIMF-mediated signaling to the phosphatidylinositol-3 kinase (PI-3K)/Akt–nuclear factor (NF)-κB pathways, and reveal the critical role of HIMF in VCAM-1 production during lung inflammation.

## MATERIALS AND METHODS

### Animal Experiments

All animal experiments followed the protocols approved by the Animal Care and Use Committee of Johns Hopkins University. Adult male C57BL/6 mice (10–12 wk old) were obtained from Jackson Laboratories (Bar Harbor, ME). Recombinant HIMF protein was produced in TREx 293 cells and the secreted HIMF in the culture medium was purified with anti-FLAG chromatography, as previously described (10). The mock purification of the culture medium obtained from control TREx 293 cells was performed to exclude the role of possible contaminants (10). To examine the effects of HIMF on VCAM-1 production *in vivo*, recombinant HIMF protein or BSA (Sigma, St. Louis, MO) was intratracheally instilled into adult mouse lungs (200 ng/animal in 40 μl saline) via a 21-gauge catheter (14). The vehicle control animals were instilled with saline (40 μl/animal). The animals were killed by halothane overdose at the time points indicated in the figure legends, and the mouse lungs were either collected for immunohistochemical staining or frozen immediately in −80°C freezer for further molecular analyses.

### Immunohistochemical Staining for VCAM-1

Lung samples were processed and immunostained as previously described (14). Briefly, the sections were incubated for 1 h with anti–VCAM-1 antibodies (1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by a 2-h incubation with goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (1: 300 dilution; Bio-Rad, Hercules, CA). DAB substrate (Dako, Carpinteria, CA) was used to generate dark brown precipitate in the cells of the tissues. The sections were examined, and images were taken with a Sony color digital DXC-S500 camera (Sony Electronics, Oradell, NJ), using Image Pro-Express software (Media Cybernetics, Silver Spring, MD).

### Western Blotting

Tissue collection, homogenization, and protein electrophoresis were performed as previously described (15). Cells were collected and proteins were extracted with 1× cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, and 1 μg/ml leupeptin). Protein (50 μg) or 40 μl of medium supernatant (for HIMF expression assays of cultured cell lines) from each sample was subjected to 4–20% precast polyacrylamide gel (Bio-Rad) electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). HIMF was detected with 1:1,000 dilution of the anti-HIMF antiserum (10). For VCAM-1 and GAPDH (Santa Cruz Biotechnology), the primary antibody dilutions were 1:500 and 1:1,000, respectively, and followed by 1:3,000 dilution of goat anti-rabbit HRP-labeled antibody (Bio-Rad). An ECL substrate kit (Amersham, Piscataway, NJ) was used for the chemiluminscent detection of the signals with autoradiography film (Amersham).

### Semiquantitative RT-PCR for VCAM-1 and HIMF

To quantify gene transcripts of VCAM-1 and HIMF in cultured cell lines, total RNA was isolated with RNeasy Mini Kit (Qiagen Inc., Valencia, CA) as specified by the manufacturer. Reverse transcription reactions were conducted with Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, IN). The PCR primers used were the following: for mouse HIMF, 5′-ATGAAGACTACAACTTGTTCCC-3′ (positions 104–125 of second exon) and 5′-TTAG GACAGTTGG CAGCAGCG-3′ (positions 419–439 of fourth exon), amplifying a 336-bp fragment; for mouse VCAM-1, 5′-CCTCACTTGCAGCAC TACGGGCT-3′ and 5′-TTTTCC AATATCCTCAATGACGGG-3′, amplifying a 442-bp fragment between positions 189 and 630; for mouse GAPDH, 5′-GCCAAGGTCATCCATGACAACTTTGG-3′ and 5′-GCCTGC TTCACCACCTTCTTGATGTC-3′, amplifying a 314-bp fragment between positions 532 and 845. PCR bands were separated on ethidium bromide–stained agarose gels. GAPDH was used to normalize the initial variations in sample concentration, and served as a control for reaction efficiency. The ratio between the amplified DNA fragments and GAPDH of each sample RNA was quantified by Phoretix 1 D software (Phoretix International Ltd., Newcastle upon Tyne, UK).

### siRNA for HIMF Knockdown

Oligonucleotides encoding short RNAi hairpin sequences specific for HIMF or *firefly* luciferase were subcloned into pSuppressor (Imgenex, San Diego, CA). Annealed oligonucleotides were cloned downstream of U6 promoter (HIMF primer 1 sequence, 5′-TCGAACTATGAACAGATG GGCCTCCGAGTACTGGGAGGCCCATCTGTTCATAGTTTTTT-3′; HIMF primer 2 sequence, 5′-CTAGAAAAAACTATGAACAGATGG GCCTCCCAGTACTCGGAGGCCCA TCTGTTCATAGT-3′; *firefly* luciferase primer 1 sequence, 5′-TCGAACGGTGGCTCCCGCTGAAT TGGAGAGTACTGTCCAATTCAGCGGGAGCCACCGTTTTTT-3′; *firefly* luciferase primer 2 sequence, 5′-CTAGAAAAAACGGTGG CTCCCGCTGAATTGGACAGTACTCTCCAATTCAGCGGGAG CCACCGT). The constructs pSuppressor-HIMF and pSuppressor-LUC were verified by sequencing analysis.

### Cell Culture and Stimulation with HIMF or LPS

SVEC 4-10, an SV40-transformed murine endothelial cell line, was obtained from the American Type Culture Collection (CRL-2181; ATCC, Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 10% FBS (Life Technologies, Inc., Gaithersburg, MD), penicillin (100 U/ml), and streptomycin (100 μg/ml). Mouse lung epithelial cell line MLE-12 was cultured as previously described (16). Both cell lines were maintained at 37°C in a humidified atmosphere of 5% $CO_2$. Confluent monolayers of SVEC 4-10 and MLE-12 were trypsinized, and $2 \times 10^5$ viable cells suspended in 2 ml of culture medium supplemented with 10% FBS were added to each well of 6-well plates. After the cell density reached 80–90%, the cell culture medium was replaced with an equal volume of medium supplemented with 0.1% FBS and 2 mM L-glutamine. Thirty-three hours later, cells were incubated in either DMEM or RPMI 1640, serum-free, for 3–4 h at 37°C, pretreated with LY294002, SB203580, PD98059, or U0126 (Calbiochem, La Jolla, CA), as indicated, then stimulated with different concentrations of HIMF protein for various periods, with or without actinomycin D (5 μg/ml; Sigma, St. Louis, MO).

### Transfection and Stable Cell Lines

HIMF cDNA vector, dominant-negative mutants of IκB kinase (IKK)α (IKKα[K44A]), IKKβ (IKKβ [K44A]), IκBα super-repressor (IκBα [S32A/S36A]), and PI-3K dominant-negative mutant (Δp85) were previously described (16–18). HIMF cDNA, RNAi, or dominant-negative mutant vectors were transfected into SVEC 4-10 and MLE-12 cells with Lipofectamine 2000 (Life Technologies). Stable cell lines expressing HIMF, named SVEC-HIMF and MLE-HIMF, along with their transfection control (vector only) cells, SVEC-Zeo and MLE-Zeo, were established based on resistance to Zeocin (Invitrogen, 400 μg/ml). HIMF overexpression was validated by both Western blotting and RT-PCR analyses.

### Dual-Luciferase Reporter Assay for VCAM-1 and NF-κB

The mouse VCAM-1 promoter-luciferase reporter construct as well as sequential deletion within the 5′-flanking sequences, pGLVCAM-1 (−3.7 luc), pGL-VCAM-1 (−1.8 luc), pGLVCAM-1 (−1.1 luc), pGLVCAM-1 (−0.7 luc), and pGLVCAM-1 (−0.3 luc) were kind gifts from Dr. Joji Ando (Department of Biomedical Engineering, Graduate School of Medicine, University of Tokyo, Tokyo, Japan) (19). The NF-κB luciferase reporter construct pNFκB-Luc, containing five tandem NF-κB binding sites (TGGGGACTTTCCGC), was purchased from Stratagene (La Jolla, CA). After the cell density reached 80–90%, cells were cotransfected with each luciferase reporter construct and renilla luciferase construct pRL-TK (Promega, Madison, WI), with or without HIMF protein stimulation. After culturing for specified periods of time, the cells were treated with passive lysis buffer according to the dual-luciferase assay manual (Promega), and luciferase activity was measured with a luminometer (Lumat LB9507; Berthold Technologies,

Bad Wildbad, Germany). The *firefly* luciferase signal was normalized to the renilla luciferase signal for each individual well.

### Phosphorylation Assay for IKK, IκBα, and Akt

SVEC 4-10 and MLE-12 cells were cultured in six-well plates with complete culture medium to 70–80% confluence. The cell culture medium was replaced with either DMEM or RPMI 1640 supplemented with 0.1% FBS and 2 mM L-glutamine for 33 h, and then changed to serum-free medium for 3–4 h at 37°C. Finally, cells were pretreated with different signal transduction inhibitors for 1 h, and incubated with HIMF protein for different time periods, as indicated. Cells were then washed once with ice-cold PBS and extracted with cell lysis buffer. Lysates (50 μg) were subjected to 4–20% precast polyacrylamide gel (Bio-Rad) electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), and probed with rabbit anti-mouse antibodies to phosphospecific and nonphosphorylated IKK, IκBα, and Akt (1:500 dilution; Santa Cruz Biotechnology), followed by 1:3,000 dilution of goat anti-rabbit HRP-labeled antibody (Bio-Rad). An ECL substrate kit (Amersham) was used for the chemiluminscent detection of the signals with autoradiography film (Amersham).

### Cell Adherence Assay

For isolation of mononuclear cells, heparinized mouse blood was diluted with PBS, and 25 ml was immediately layered over 15 ml Ficoll-Paque (Amersham) and centrifuged (400 × *g*, 40 min, 22°C). The mixed mononuclear cell band was removed by aspiration, washed with PBS, and incubated for 2 h with anti-CD45 antibody (Santa Cruz Biotechnology), followed by a 1-h incubation with goat anti-rabbit antibody conjugated with fluorescein (1: 300 dilution; Vector Laboratories, Inc., Burlingame, CA). The labeled cells were counted and used in the adhesion assay. Confluent monolayers of SVEC 4-10 and MLE-12 were trypsinized, and $1 \times 10^5$ viable cells suspended in 1 ml of culture medium supplemented with 10% FBS were added to each well of the 12-well plates. After the cell density reached 80–90%, the cell culture medium was replaced with an equal volume of medium supplemented with 0.1% FBS and 2 mM L-glutamine. Twenty-four hours later, cells were pretreated as indicated, and stimulated with HIMF protein for 24 h. Then, $1 \times 10^5$ mononuclear cells were added to each well, and the plates were incubated for 30 min at 37°C. The nonadherent mononuclear cells were removed by carefully washing the cells twice with medium. Finally, the number of adherent mononuclear cells was determined by counting five random high-power fields under a fluorescence microscope (Leitz Fluovert; Leitz, Rockleigh, NJ), and expressed as cell numbers/high-power field. For VCAM-1–blocking studies, SVEC 4-10 and MLE-12 cells were preincubated with anti–VCAM-1 antibody, control rabbit IgG or signal transduction inhibitors, respectively, for 30 min at 37°C with 5% $CO_2$. SVEC 4-10 and MLE-12 cells were washed once with Hank's balanced salt solution without calcium and magnesium (HBSS, Life Technologies), and the mononuclear cells were added and incubated as described above.

### Statistical Analysis

Unless otherwise stated, all data were shown as mean ± SEM. Statistical significance ($P < 0.05$) was determined by *t* test or ANOVA, followed by assessment of differences using SigmaStat 2.03 software (Jandel, Erkrath, Germany).

## RESULTS

### HIMF Enhances VCAM-1 Expression in Mouse Lungs

To test the hypothesis that HIMF participates in regulation of VCAM-1 expression, we first tested whether administration of HIMF would enhance VCAM-1 expression in the lung. We instilled HIMF protein intratracheally into adult mouse lungs and performed immunohistochemical staining. As shown in Figure 1A, HIMF protein instillation resulted in a significant increase of VCAM-1 production in vascular endothelial, alveolar type II, and airway epithelial cells. In contrast, no significant VCAM-1 expression was observed in control mouse lungs treated with either saline or BSA. Western blotting experiments showed that, after intracheal instillation of HIMF for 6 h and 24 h, VCAM-1 expression was significantly enhanced in the lung tissues (Figure 1B). These results provide the first evidence that connect HIMF to VCAM-1 expression in mouse lungs.

### Treatment of HIMF Increases VCAM-1 Expression in Mouse Endothelial and Lung Epithelial Cells

To investigate how HIMF modulates VCAM-1 expression, we examined the level of VCAM-1 protein and mRNA in HIMF-treated mouse endothelial and lung epithelial cell lines, SVEC 4-10 and MLE-12. As shown in Figure 2A, addition of recombinant HIMF into cell culture medium induced VCAM-1 protein production in both SVEC 4-10 and MLE-12 cells in an HIMF dose–dependent manner. This result was also confirmed by RT-PCR (Figure 2B). Time-course studies showed that VCAM-1 production appeared at 6 h after administration of HIMF (40 nmol/liter), and was sustained until 24 h after administration (Figures 2C and 2D). To validate that HIMF enhances VCAM-1 expression, we established mouse endothelial and lung epithelial cell lines, SVEC-HIMF and MLE-HIMF, that stably express HIMF. As shown in Figures 3A and 3B, SVEC-HIMF and MLE-HIMF cells overexpressed HIMF mRNA and protein as compared with their parent and transfection controls. More importantly, the VCAM-1 mRNA and protein levels of SVEC-HIMF and MLE-HIMF were also increased significantly compared with those of their control counterparts (Figures 3A and 3B). These results clearly demonstrate that HIMF induces VCAM-1 production.

### Knockdown of HIMF Attenuated LPS-Induced VCAM-1 in Mouse Endothelial and Lung Epithelial Cells

LPS has been considered as the principal component in induction of VCAM-1 expression during lung inflammation. To further explore the role of HIMF in lung inflammation, we first performed knockdown of HIMF expression by RNA interference, then observed the changes in LPS-induced VCAM-1 upregulation in both endothelial and lung epithelial cells. As shown in Figures 4A and 4B, LPS induced extensive HIMF expression accompanied by upregulation of VCAM-1 in SVEC 4-10 and MLE-12 cells. When these cells were transfected with HIMF RNAi vector, the HIMF expression was abolished to a great extent. Moreover, knockdown of HIMF expression attenuated the LPS-induced VCAM-1 upregulation. However, transfection of cells with RNAi vector for *firefly* luciferase (nonfunctional control) or empty vector did not attenuate HIMF expression and LPS-induced VCAM-1 production, which indicates the specificity of the HIMF RNAi construct (Figures 4A and 4B). In view of the above findings that HIMF promotes VCAM-1 expression *in vivo* and *in vitro*, these results indicate that HIMF plays an important role in lung inflammation.

### HIMF Enhances Transcription of VCAM-1 Rather than Its mRNA Stability

To understand how HIMF regulates VCAM-1 expression, we used a construct, pGLVCAM-1 (−3.7 luc), that contains a VCAM-1 promoter–driven luciferase reporter gene. As shown in Figure 5A, incubation of pGLVCAM-1–transfected SVEC 4-10 and MLE-12 cells with HIMF protein resulted in significant increases of transcripts of VCAM-1 promoter–driven luciferase in an HIMF dose–dependent manner. Because modulation of mRNA stability has been reported as an important post-transcriptional mechanism that controls VCAM-1 gene expression (20), it is possible that HIMF enhances VCAM-1 expression by inducing factor(s) that contribute to VCAM-1 mRNA stability. To test this possibility, we examined the effects of HIMF on

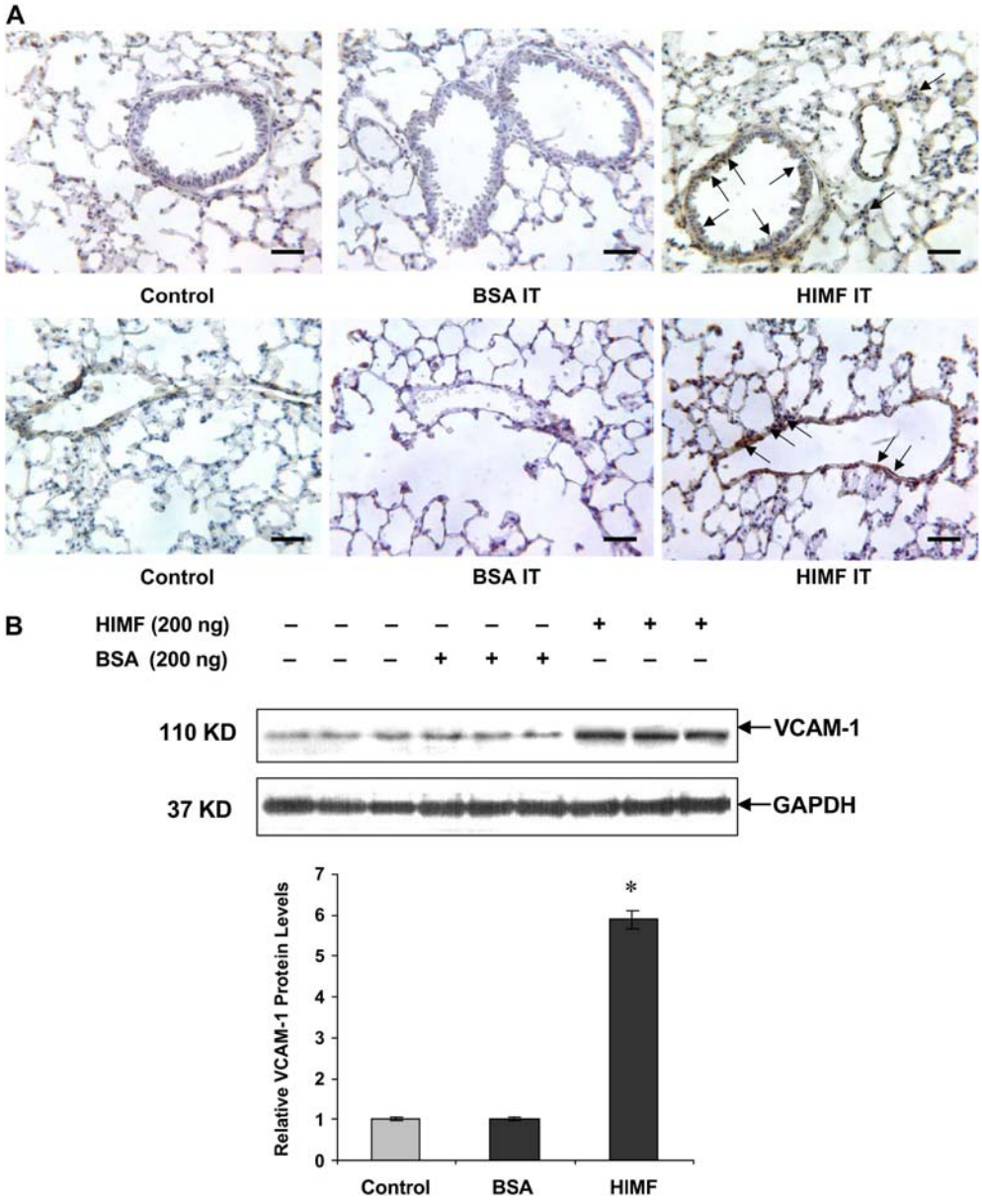


***Figure 1.*** HIMF enhances VCAM-1 expression in mouse lungs. Recombinant HIMF protein or BSA was intratracheally instilled into adult mouse lungs (200 ng/animal in 40 μl saline, $n = 6$). The vehicle controls were instilled with saline (40 μl/animal; $n = 3$). The mouse lungs were collected after 6 and 24 h. (*A*) Results of immunohistochemical staining indicated that instillation of HIMF protein, but not BSA, resulted in a significant increase of VCAM-1 production, mainly localized to alveolar type II and airway epithelial cells (*right upper panel*, *arrows*), and vascular endothelial cells (*right lower panel*, *arrows*). *Scale bars*: 100 μm. (*B*) Western blot with proteins from lung homogenates indicated that VCAM-1 production was enhanced in HIMF-instilled, but not in BSA- or saline-instilled mouse lungs. *Asterisk* indicates a significant increase compared with control mouse lungs instilled with saline only ($P < 0.05$).

VCAM-1 mRNA stability by using a transcription inhibitor, actinomycin D. If HIMF affects the stability of VACM-1 mRNA, blocking transcription by actinomycin D should allow an observation of stabilized VCAM-1 mRNA in the cell. However, pretreatment of SVEC 4-10 and MLE-12 cells with HIMF protein and then actinomycin D did not prevent the existing VCAM-1 mRNA from degradation (Figures 5B and 5C), suggesting that HIMF enhances VCAM-1 expression in mouse endothelial and epithelial cells through increasing its transcription, rather than strengthening its mRNA stability.

### Activation of NF-κB Is Essential for HIMF-Induced VCAM-1 Production

To search for the putative transcription factor(s) involved in HIMF-induced VCAM-1 production, we used sequentially deleted 5′-flanking sequences of mouse VCAM-1 promoter (Figure 6A). The sequences TGGGTTTCCC at −73 bp and AGGGATTTCCC at −58 bp are identical to the consensus sequence (GGG R[C/A/T]TYYCC) of the NF-κB binding site (19). The sequence TGACTCA at both −481 and −461 bp matches the AP-1 consensus sequence perfectly (19). A sequence (CGTCA) with homology to cAMP response element was also identified at position −1420 (19). As shown in Figure 5B, treatment of HIMF induces VCAM-1 promoter–luciferase reporter activity significantly in transfected SVEC-HIMF and MLE-HIMF cells. The highest VCAM-1 luciferase activity was measured from a deletion construct pGLVCAM-1 (−0.3 luc) that contains 2 NF-κB binding sites within 329 bp of VCAM-1 promoter, whereas another deletion construct, pGLVCAM-1 (−0.7 luc) exhibited lower luciferase activity (Figure 6B). Other VCAM-1 promoter deletions with sequences longer than pGLVCAM-1 (−0.7 luc) showed a similar lower level of luciferase activity as pGLVCAM-1 (−0.7 luc) (Figure 5B). These observations suggest that a negative regulatory element might exist between the −0.7 and −0.3 regions of the VCAM-1 promoter. It has been reported that NF-κB regulates VCAM-1 expression (21). It is unknown, though, whether HIMF enhances VCAM-1 expression through NF-κB pathway. To assess this possibility, we used

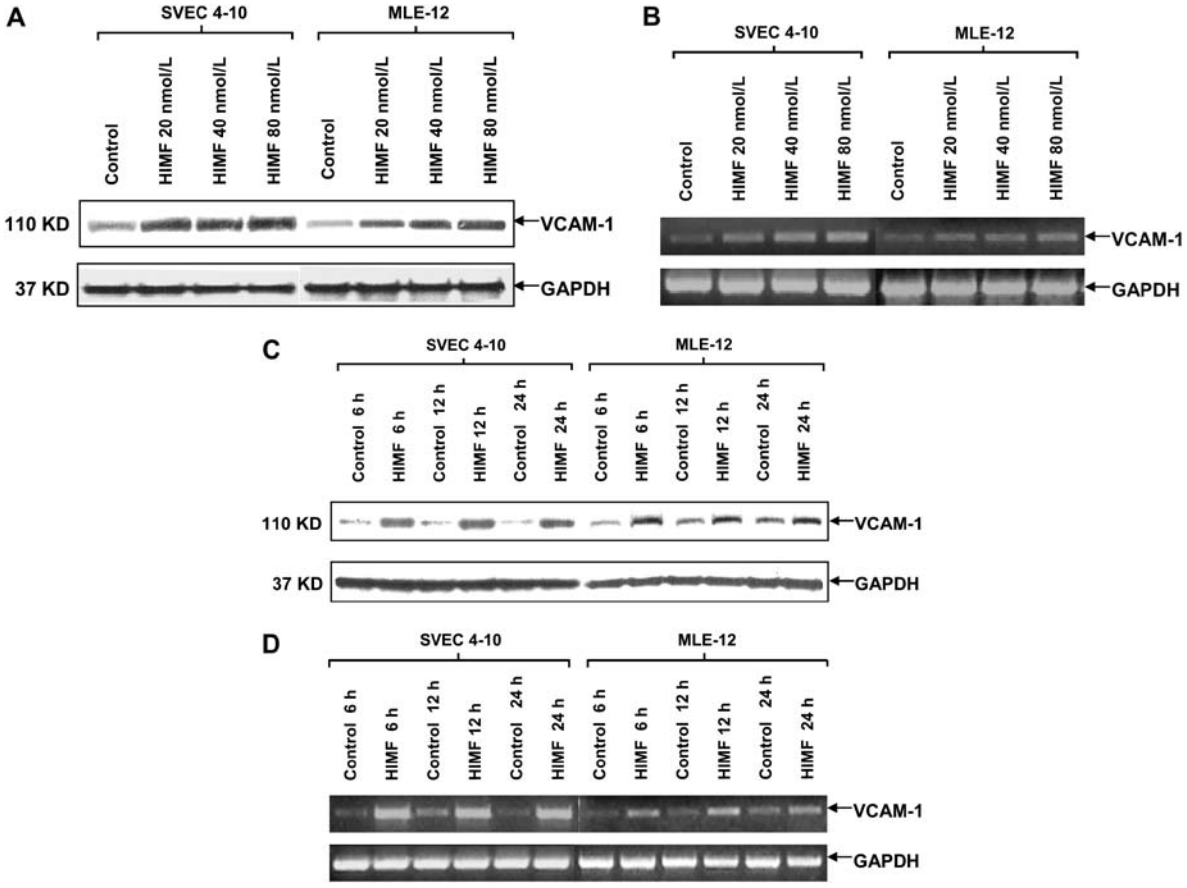


*Figure 2.* HIMF induces VCAM-1 expression in mouse endothelial and lung epithelial cell lines. SVEC 4-10 and MLE-12 cells were starved with culture medium supplemented with 0.1% FBS and 2 mM L-glutamine. After 33 h, cells were treated with different concentrations of HIMF for various periods, as indicated. Western blot and semiquantitative RT-PCR were performed for VCAM-1 expression. Incubation of SVEC 4-10 and MLE-12 cells with 20, 40, and 80 nmol/liter of HIMF protein for 24 h resulted in increased VCAM-1 protein and mRNA levels in a dose-dependent manner (*A* and *B*). Time-course study indicated that HIMF (40 nmol/liter)-induced VCAM-1 production started at 6 h, and was sustained until 24 h (*C* and *D*). Triplicate experiments were performed with essentially identical results.

NF-κB luciferase reporter assay combined with transfection of several dominant-negative mutants in NF-κB pathway, IKKα (K44A), IKKβ (K44A), and IκBα (S32A/S36A) in SVEC 4-10 and MLE-12 cells. As shown in Figure 5C, NF-κB activities in SVEC-HIMF and MLE-HIMF cells were significantly higher than those of their control counterparts, indicating HIMF-induced activation of NF-κB in these cells. Furthermore, incubation of SVEC 4-10 and MLE-12 cells with HIMF protein resulted in an increase of NF-κB activity in 6 h, and this increase was sustained until 24 h (Figure 7A). In response to HIMF, IKK was phosphorylated, which in turn phosphorylates IκBα, leading to NF-κB activation in both SVEC 4-10 and MLE-12 cells (Figure 7B). In contrast, transfection of dominant-negative mutants IKKα (K44A), IKKβ (K44A), and IκBα (S32A/ S36A) abolished HIMF-induced NF-κB activation, leading to suppression of VCAM-1 transcription (Figure 7C) and protein expression (Figure 7D). Together, these results demonstrate that activation of transcription factor NF-κB is essential for HIMF-induced VCAM-1 upregulation, and thus connected HIMF signaling to NF-κB pathway.

### PI-3K/Akt Pathway Is Involved in HIMF-Induced NF-κB Activation and VCAM-1 Production

Our previous study showed that HIMF activates the PI-3K/Akt pathway in pulmonary microvascular smooth muscle cells (10). It is also known that PI-3K/Akt pathway participates in NF-κB activation induced by cytokines (22, 23). Because HIMF, as an extracellular ligand, may function in a manner similar to a cytokine, we also tested whether PI-3K/Akt participates in HIMF-mediated NF-κB activation. As shown in Figure 8A, HIMF strongly induced Akt phosphorylation at Ser473 and Thr308. The Akt activation was observed at 30 min of HIMF treatment, and the activation was sustained for 360 min. The PI-3K inhibitor, LY294002 (10 μmol/liter), inhibited HIMF-activated Akt phosphorylation (Figure 8B). However, incubation of cells with SB203580 (5 μmol/liter), PD098059 (5 μmol/liter), or U0126 (5 μmol/liter), inhibitors against p38 and ERK1/2 MAPK pathways, respectively, had no effect on HIMF-induced Akt phosphorylation (Figure 8B). In addition, transfection of Δp85, a dominant-negative mutant of PI-3K, into SVEC 4-10 and MLE-12 cells, abolished HIMF-induced phosphorylation of IKK and IκBα (Figure 8C), and the subsequent NF-κB activation. These observation suggest that PI-3K/Akt pathway functions upstream of NF-κB in responding to HIMF signals. Transfection of Δp85 also blocked HIMF-induced transcription of VCAM-1 (Figure 8D). Similarly, PI-3K inhibitor LY294002 specifically suppressed HIMF-induced VCAM-1 production in SVEC 4-10 and MLE-12 cells (Figure 8E). Together, these data suggest that the PI-3K/Akt pathway is involved in HIMF-mediated NF-κB activation and the subsequent VCAM-1 production.

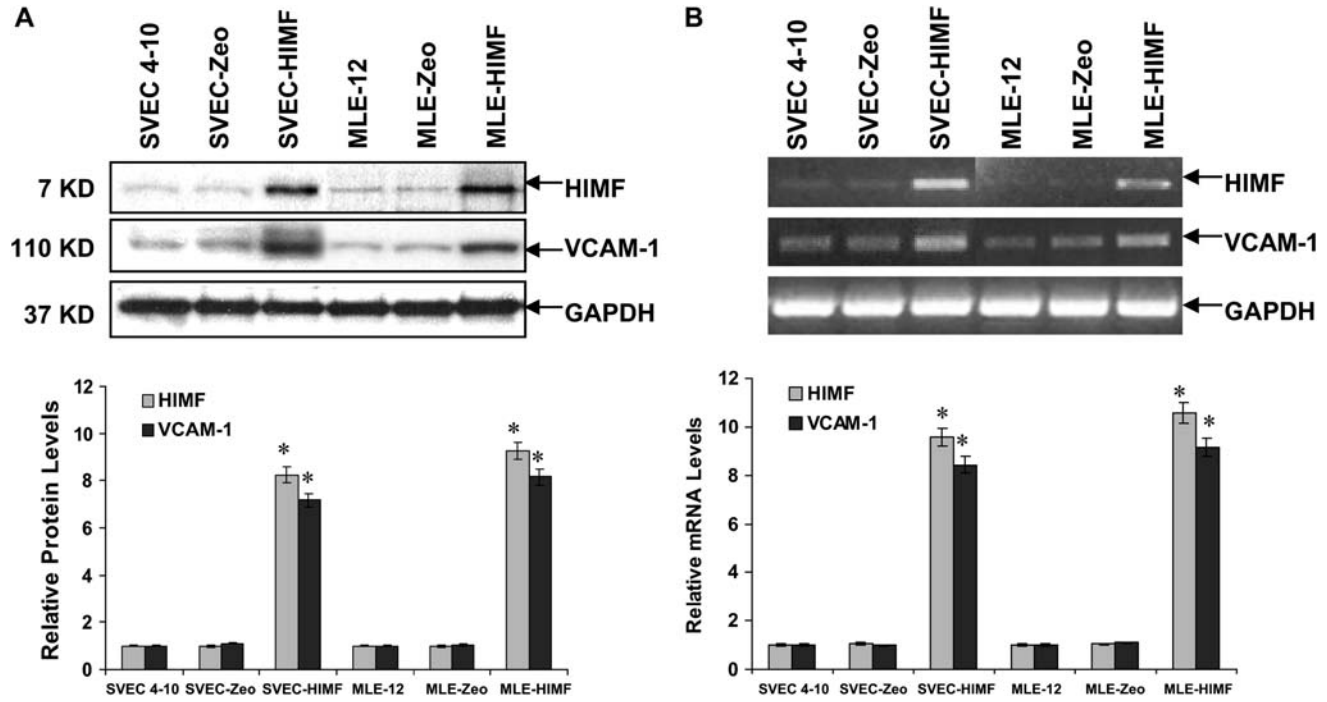


*Figure 3.* HIMF overexpression in mouse endothelial and epithelial cell lines. SVEC 4-10 and MLE-12 cells were transfected by HIMF cDNA or control vector with Lipofectamine 2000. Stable cell lines, SVEC-HIMF and MLE-HIMF, along with their transfection control cells, SVEC-Zeo and MLE-Zeo, were selected based on resistance to Zeocin (400 μg/ml). Western blot (*A*) and RT-PCR (*B*) demonstrated that SVEC-HIMF and MLE-HIMF cells have increased HIMF protein and mRNA levels compared to their parent and transfection counterparts. Their VCAM-1 expression was also increased significantly compared with SEVC 4-10 and MLE-12 cells. *Asterisk* indicates a significant increase compared with parent controls ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

### HIMF-Induced VCAM-1 Upregulation Promotes Adherence of Mononuclear Cells to SVEC 4-10 and MLE-12 Cells

One of the prominent functions of VCAM-1 is to mediate the adhesion of mononuclear cells by interaction with its counter-receptor, VLA4, and thus enhances inflammation (4, 5). To elucidate the effects exerted by HIMF-induced VCAM-1, we isolated the mononuclear cells from peripheral blood, and examined their adherence to HIMF-treated mouse endothelial and lung epithelial cells. HIMF stimulation resulted in an increase of adherence of mononuclear cell to SVEC 4-10 and MLE-12 cells (Figure 9A), and such adherence was blocked by anti–VCAM-1 antibody. When SVEC 4-10 and MLE-12 cells were pretreated with PI-3K inhibitor LY294002 (10 μmol/liter) before HIMF incubation, the adherent mononuclear cells were significantly reduced (Figure 9B). In contrast, SB203580 (5 μmol/liter), PD098059 (5 μmol/liter), or U0126 (5 μmol/liter) did not abolish HIMF-induced mononuclear cell adherence to SVEC 4-10 and MLE-12 cells (Figure 9B). These results indicate that VCAM-1 upregulation induced by HIMF promotes the adherence of mononuclear cells to endothelial and lung epithelial cells, which is one of the most important events in lung inflammatory processes.

## DISCUSSION

VCAM-1 was first identified as an adhesion molecule induced in endothelial cells by inflammatory cytokines IL-1 and TNF-α, or by LPS (6, 7). In a murine model of ovalbumin-induced pulmonary inflammation, VCAM-1 was found to be prominently expressed on lung vascular tissues, which has important roles in antigen-induced recruitment of T cells and eosinophils (24). To date, there is only limited information available about the pattern and the signaling to VCAM-1 expression in alveolar epithelial cells. Cunningham and colleagues first investigated the expression of adhesion molecules VCAM-1, LFA-3, and B7 by alveolar epithelium (25). These molecules are not detected on the surface of isolated human type II pneumocytes (25). VCAM-1 is uniformly expressed on the vessels of the frozen sections of lung specimens, and was occasionally observed on the alveolar tissues (25). However, recent studies have demonstrated that constitutive VCAM-1 expression was enhanced by rhinovirus infection of alveolar epithelial cell lines (26). In addition, increased VCAM-1 expression has been noted on isolated human alveolar epithelial cells stimulated with TNF-α (27). These results provide evidence of VCAM-1 expression in stimulated alveolar epithelial cells. In the present study, we found that HIMF induced VCAM-1 production in mouse lung tissues and cultured endothelial and alveolar epithelial cell lines through enhancing its transcription, but without affecting its mRNA stability. The process is dependent upon activated PI-3K/Akt and NF-κB. These results suggest that HIMF may play critical roles in VCAM-1 production and lung inflammation via the PI-3K/Akt–NF-κB pathways.

HIMF, also known as FIZZ1 in a lung allergic inflammation mouse model (11), belongs to a novel class of cysteine-rich secreted proteins known as resistin-like molecules (12). During allergic pulmonary inflammation, HIMF/FIZZ1 expression markedly increases in the bronchial mucosal epithelial cells, and it also appears in alveolar type II cells, but not in alveolar macrophages (11). The effects of HIMF/FIZZ1 on cell adhesion molecules involved in lung inflammation, however, remain largely unknown.

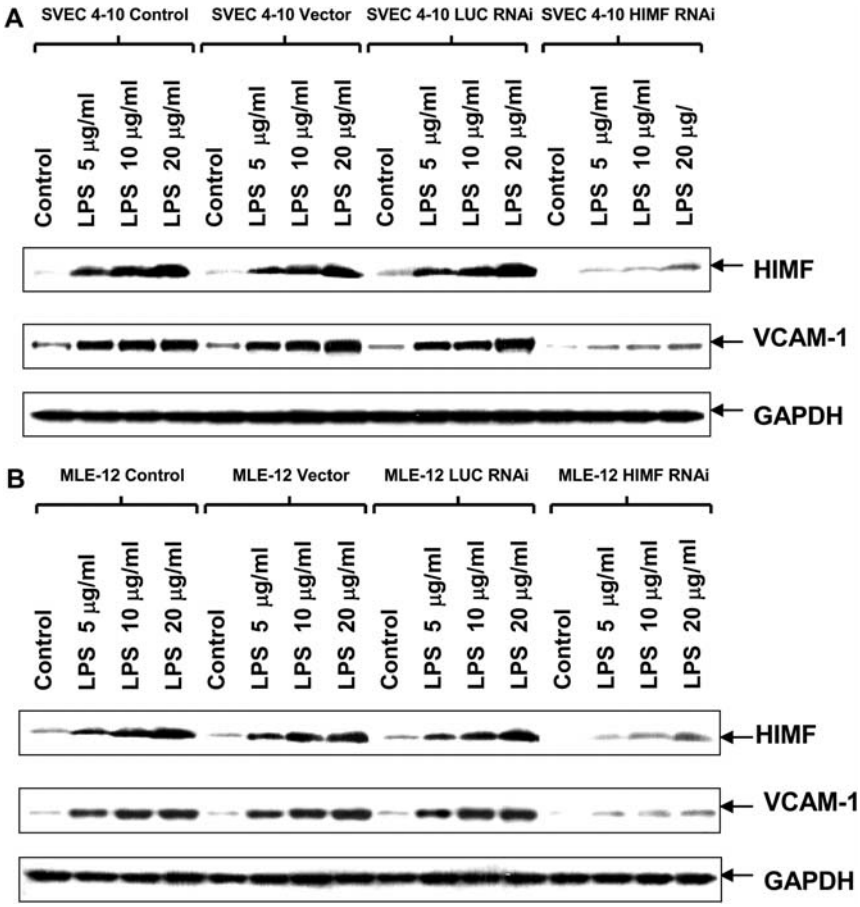


*Figure 4.* Knockdown of HIMF attenuated LPS-induced VCAM-1 in mouse endothelial and lung epithelial cells. Confluent monolayers of SVEC 4-10 and MLE-12 were transfected with HIMF RNAi, LUC RNAi, or empty vectors, respectively. After 24 h, the cells were stimulated with different concentrations of LPS for various periods, as indicated. Western blot demonstrated that LPS induced intensive HIMF expression accompanied by upregulation of VCAM-1 in SVEC 4-10 (*A*) and MLE-12 (*B*) cells; however, transfection of cells with HIMF RNAi vector, but not LUC RNAi or empty vectors, abolished the HIMF expression to a great extent. Moreover, knockdown of HIMF expression attenuated the LPS-induced VCAM-1 upregulation. Triplicate experiments were performed with essentially identical results.

We have discovered that HIMF is upregulated in alveolar epithelial and endothelial cells during LPS-induced lung injury. Such injury is accompanied by extensive inflammatory cell sequestration in the lung parenchyma (data not shown). Because it is known that VCAM-1 enhances cell adhesion, we postulate that HIMF may modulate VCAM-1 expression. To obtain direct evidence for this hypothesis, we instilled HIMF protein intratracheally into adult mouse lungs (Figure 1). We found that VCAM-1 expression was significantly enhanced by HIMF stimulation in vascular endothelial, alveolar type II, and airway epithelial cells. These *in vivo* findings were confirmed in both HIMF-treated endothelial and lung epithelial cells (Figure 2), or lung cell lines that stably express HIMF (Figure 3). Furthermore, small interference (siRNA)-mediated knockdown of HIMF expression attenuated LPS-induced VCAM-1 expression in both endothelial and lung epithelial cells, indicating that HIMF plays a critical role in lung inflammation under normoxic conditions. However, our preliminary work indicates that hypoxia downregulates the VCAM-1 expression in both mouse endothelial and epithelial cells, and stable transfection of HIMF does not prevent hypoxia-mediated VCAM-1 decrease (data not shown). The mechanisms are unknown; however, it is conceivable to speculate that hypoxia-induced changes mediating VCAM-1 downregulation overcomes HIMF-induced VCAM-1 upregulation. Further investigation to identify these changes is warranted to clarify this issue in the future.

Up to now, the organization of regulatory elements required for cytokine-induced expression of VCAM-1 has only been partially defined (28, 29). In an effort to understand the transcription factors controlling the VCAM-1 response to HIMF, we conducted transient transfection experiments with segments of VCAM-1 5′-flanking promoter sequence coupled to a luciferase reporter (19). We found that a small region upstream of the transcription start site, which contains a tandem binding site for NF-κB, is capable of directing highest HIMF-induced VCAM-1 expression. This finding is consistent with the previous finding that activation of VCAM-1 requires two tandem binding sites for NF-κB, located in the basal VCAM-1 promoter at positions −73 and −58, both of which are necessary for cytokine-mediated transcriptional response (28, 30). Mutation of either of these elements abolishes cytokine responsiveness (28). Studies of human VCAM-1 promoter also suggest that TNF-α–mediated activation of VCAM-1 transcription in endothelial cells is dependent, at least in part, on the activation of NF-κB transcription factors (28). In addition, Kawanami and colleagues found that C-reactive protein induces VCAM-1 expression in an NF-κB–dependent mechanism, which further emphasizes the importance of NF-κB in VCAM-1 gene expression (31). In this study, we found that, in SVEC 4-10 and MLE-12 cells, HIMF induces NF-κB activation in a dose-dependent manner. When HIMF-induced NF-κB activation was blocked by transfection of dominant-negative mutants in the NF-κB pathway, IKKα (K44A), IKKβ(K44A), and IκBα (S32A/S36A), the VCAM-1 promoter activity and its expression were also decreased accordingly. These results demonstrate that activation of NF-κB is essential for HIMF-induced VCAM-1 expression in mouse endothelial and epithelial cells, and, for the first time, connect HIMF signaling to the NF-κB pathway.

NF-κB is a dimeric, ubiquitously expressed transcription factor that plays a critical role in regulating inducible gene

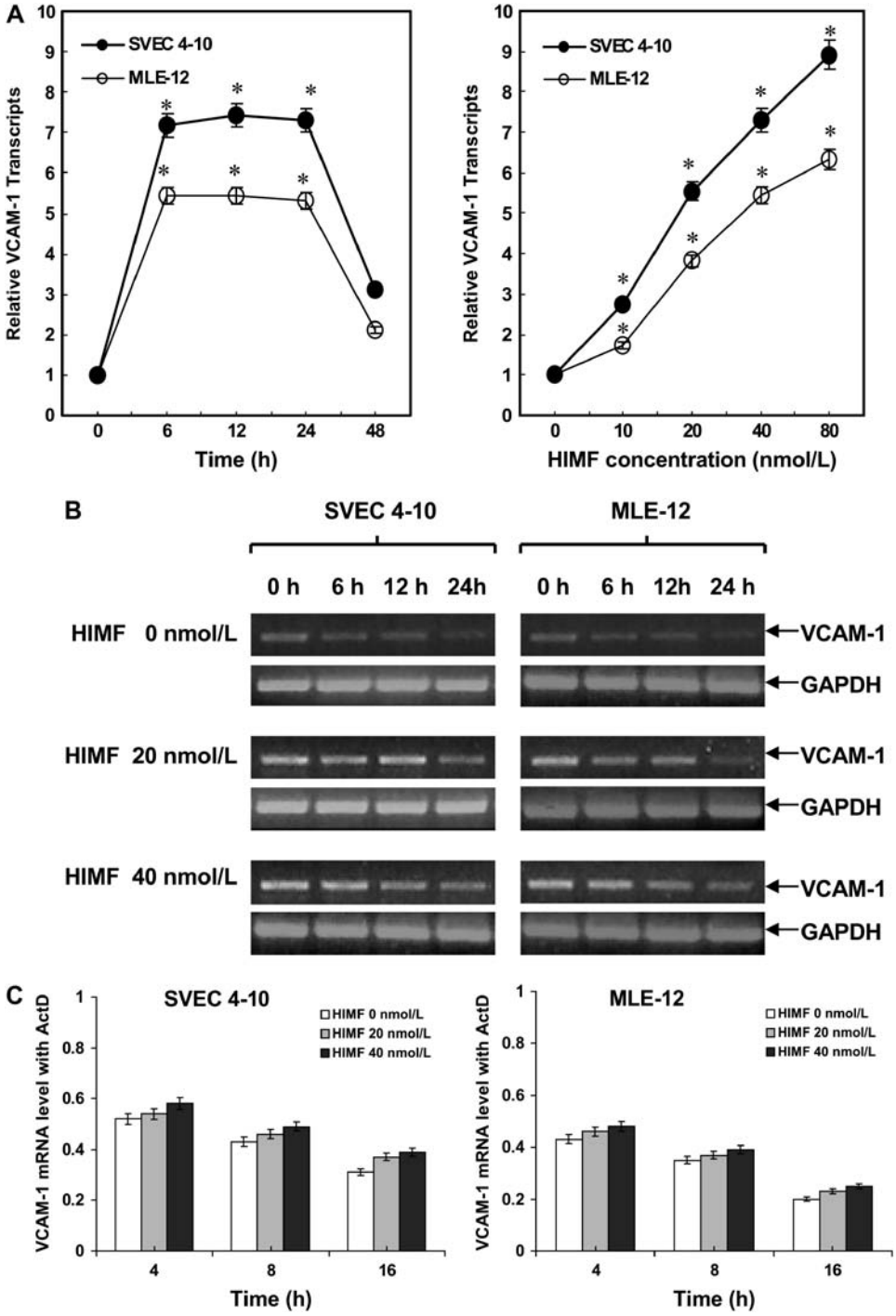


*Figure 5.* HIMF increases the transcription rather than mRNA stability of VCAM-1 in SVEC 4-10 and MLE-12 cells. (*A*) SVEC 4-10 and MLE-12 cells were cotransfected with pGLVCAM-1 (−3.7 luc) and pRL-TK. After 24 h, the cells were incubated with HIMF protein, as indicated. Cells were then lysed with passive lysis buffer, and luciferase activity was measured according to the dual-luciferase assay manual. The *firefly* luciferase signal was normalized to the renilla luciferase signal for each individual well. The time-course study showed that the transcription of VCAM-1 induced by HIMF (40 nmol/liter) started at 6 h and persisted until 24 h. After incubation with 10–80 nmol/liter of HIMF for 24 h, transcription of VCAM-1 in SVEC 4-10 and MLE-12 cells was enhanced in a dose-dependent manner. (*B* and *C*) SVEC 4-10 and MLE-12 were treated with different concentrations of HIMF, and incubated with 5 μg/ml of actinomycin D for 4, 8, and 16 h. RT-PCR showed that HIMF did not prevent actinomycin D–facilitated VCAM-1 messenger degradation in SVEC 4-10 and MLE-12 cells. *Asterisks* indicate a significant increase from SVEC 4-10 or MLE-12 controls untreated with HIMF ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

expression in inflammatory responses (32). In resting cells, NF-κB is normally sequestered in the cytoplasm through its interaction with the IκB (inhibitory of NF-κB) family of inhibitory proteins (33). In response to external stimuli, IκB proteins undergo rapid phosphorylation on specific serine residues. Phosphorylation of IκBα on serines 32 and 36, and of IκBβ on serines 19 and 23, facilitates their ubiquitination on neighboring lysine residues, thereby targeting these proteins for rapid degradation by the proteosome (32). After the degradation of IκB, NF-κB is released and is free to translocate to the nucleus and to activate target genes. A key regulatory step in this pathway is the activation of a high molecular weight IKK complex, in which catalysis is carried out by multiple kinases, including IKKα and IKKβ (33). In the present study, we found that phosphorylation of IKK was induced by administration of HIMF. Moreover, transfection of dominant-negative mutants of IKKα and IKKβ inhibited HIMF-induced NF-κB activation. In addition, dominant-negative mutant of IKKβ exhibited more pronounced inhibitory effects on HIMF-induced NF-κB activation than that of IKKα. Our observations are consistent with data obtained from gene targeting studies that suggest IKKβ, but not IKKα, plays a major role in the induction of NF-κB activity in response to inflammatory stimuli (34).

Substantial progress has been made in understanding the signal transduction pathways regulating activation of NF-κB in

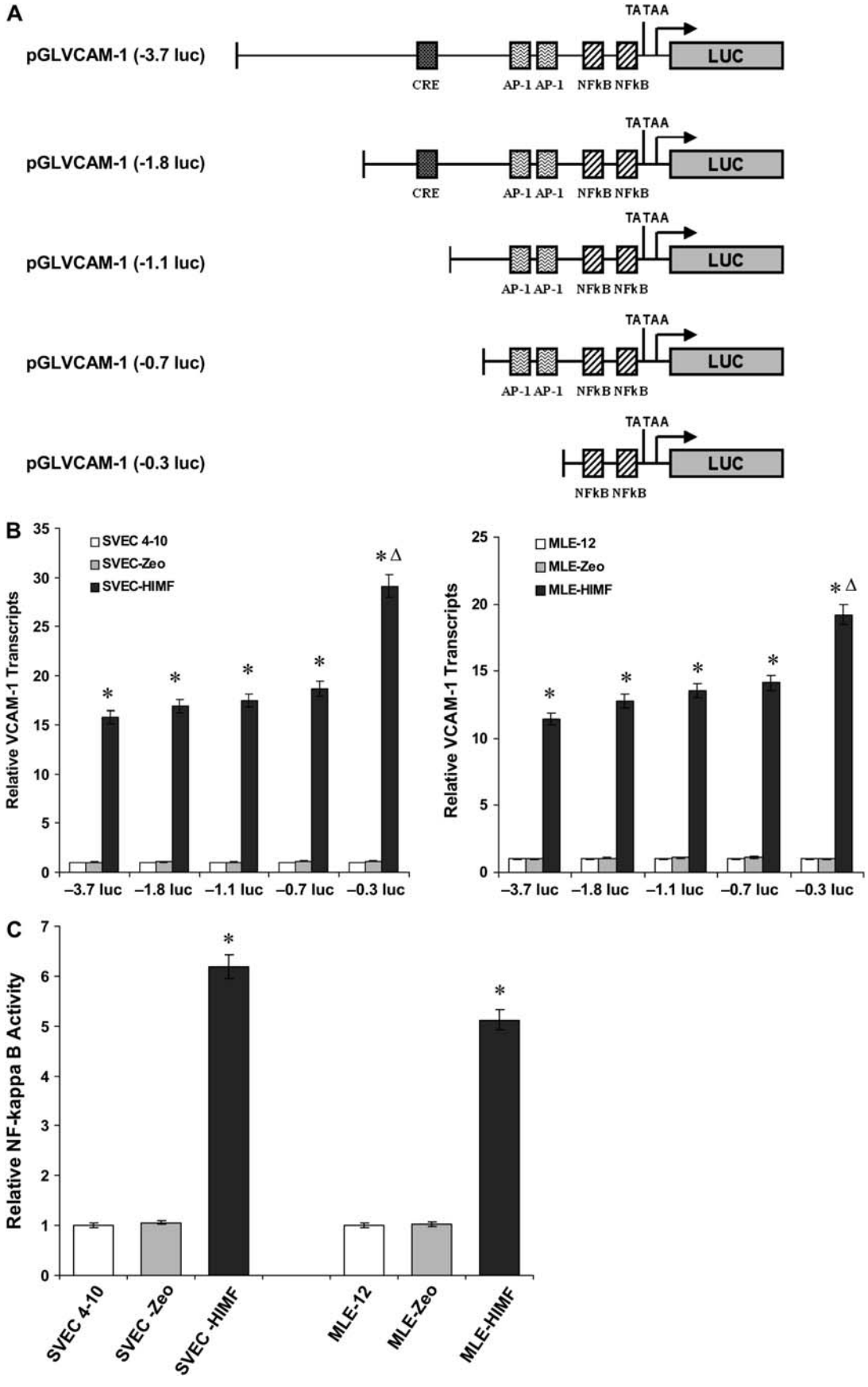


*Figure 6.* Sequential promoter deletion assays for HIMF-induced VCAM-1 expression in SVEC 4-10 and MLE-12 cells. SVEC-HIMF and MLE-HIMF, and their control counterpart cells, were cotransfected with pRL-TK and luciferase reporter constructs for either VCAM-1 (*A*) or NF-κB. After 24 h, cells were lysed with passive lysis buffer, and luciferase activity was measured according to the dual-luciferase assay manual. The *firefly* luciferase signal was normalized to the renilla luciferase signal for each individual well. (*B*) HIMF overexpressing cell lines, SVEC-HIMF and MLE-HIMF, had higher VCAM-1 transcription activity than their control counterparts. The highest VCAM-1 activity was observed with transfection of pGLVCAM-1 (−0.3 luc) construct, which contains 2 NF-κB binding sites within −329 bp of VCAM-1 promoter. (*C*) Dual-luciferase assays showed that SVEC-HIMF and MLE-HIMF had higher NF-κB activity than their control counterparts. *Asterisks* indicate a significant increase from SVEC 4-10 or MLE-12 parent controls ($P < 0.05$). *Triangles* indicate a significant increase from SVEC-HIMF or MLE-HIMF transfected with pGLVCAM-1(−3.7 luc) ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

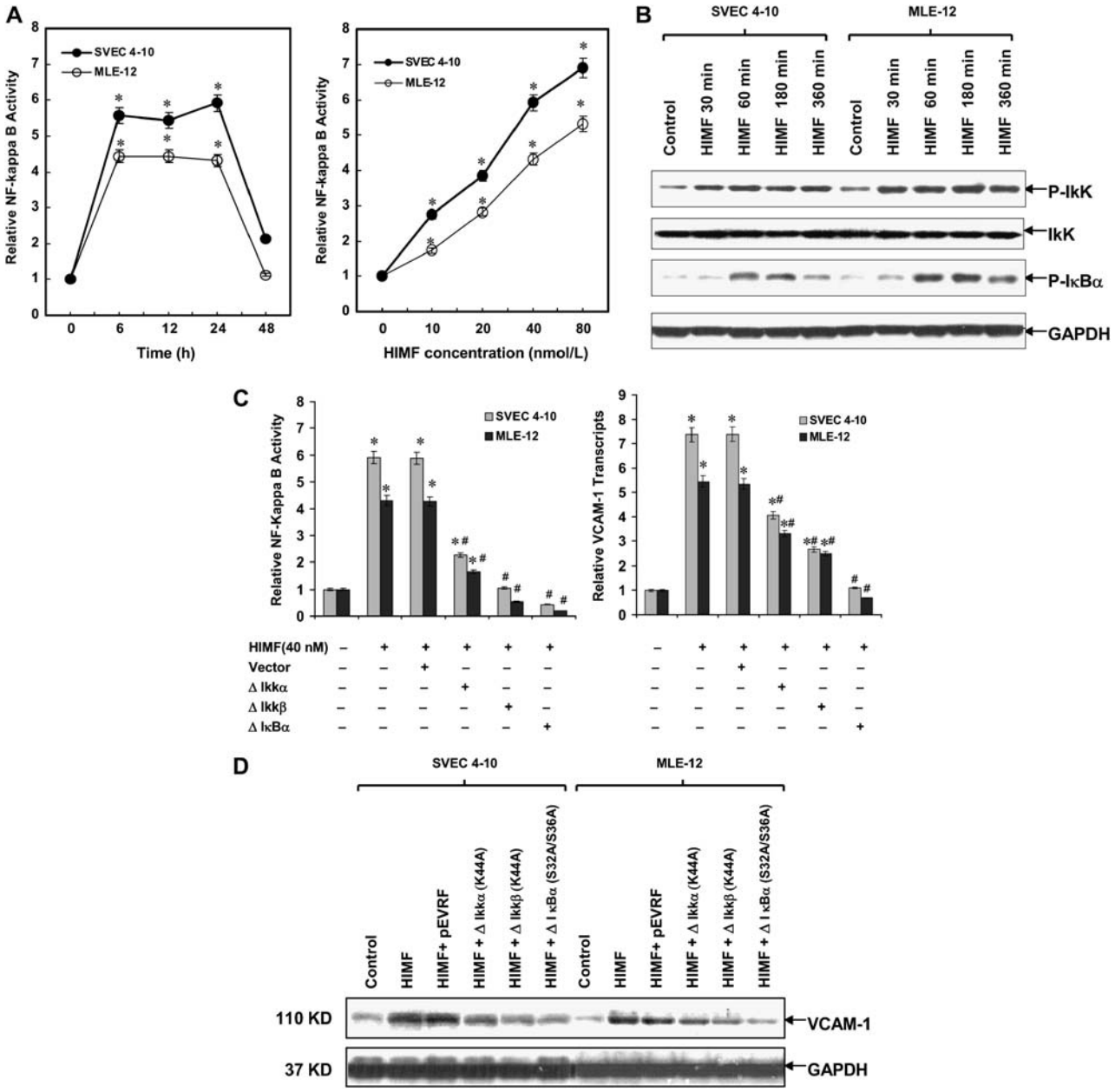


***Figure 7.*** Activation of NF-κB is essential for HIMF-induced VCAM-1 expression. SVEC 4-10 and MLE-12 cells were cotransfected with pNFκB-luc, dominant-negative mutants, and pRL-TK. The cells were stimulated with HIMF protein for various periods, as indicated. (*A*) Dual-luciferase assay indicated that the NF-κB activity of SVEC 4-10 and MLE-12 cells induced by HIMF (40 nmol/liter) started at 6 h and was sustained for 24 h, and that this response was in a dose-dependent manner (data shown represent cells treated with HIMF for 24 h). (*B*) Western blot results indicated that HIMF (40 nmol/liter) induced phosphorylation of IKK and IκBα, important kinases upstream of NF-κB activation, in SVEC 4-10 and MLE-12 cells. (*C*) Transfection of SVEC 4-10 and MLE-12 cells with dominant-negative mutants IKKα (K44A), IKKβ (K44A), and IκBα (S32A/S36A) for 24 h abolished HIMF (40 nmol/liter)-induced NF-κB activity and VCAM-1 transcription activity. (*D*) Western blot results further confirmed the abolished VCAM-1 production by these dominant-negative mutants. *Asterisks* indicate a significant increase from SVEC 4-10 or MLE-12 controls untreated with HIMF ($P < 0.05$). *Pound symbols* indicate a significant decrease from SVEC 4-10 or MLE-12 cells treated with HIMF only ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

response to proinflammatory cytokines, such as TNF-α and IL-1β (32). PI-3K stimulates NF-κB activation through a downstream serine/threonine kinase Akt, as shown in TNF-α– and platelet-derived growth factor–induced NF-κB activation (22, 23). PI-3K is a lipid kinase that is composed of two polypeptides: a p85 regulatory subunit, and a p110 catalytic subunit. This kinase is activated by a large spectrum of cytokines, hormones, and growth factors (35). Our results demonstrate that HIMF induced Akt phosphorylation in both SVEC 4-10 and MLE-12 cells. The time course of the induced Akt activation is compatible with that of NF-κB activation in HIMF-stimulated cells. Pretreatment of cells with a PI-3K–specific inhibitor, LY294002, attenuated HIMF-induced Akt phosphorylation. Furthermore, transfection of Δp85, the dominant-negative mutant of PI-3K, into cells blocked HIMF-induced phosphorylation of IKK and IκBα, resulting in inhibition of NF-κB activation and VCAM-1

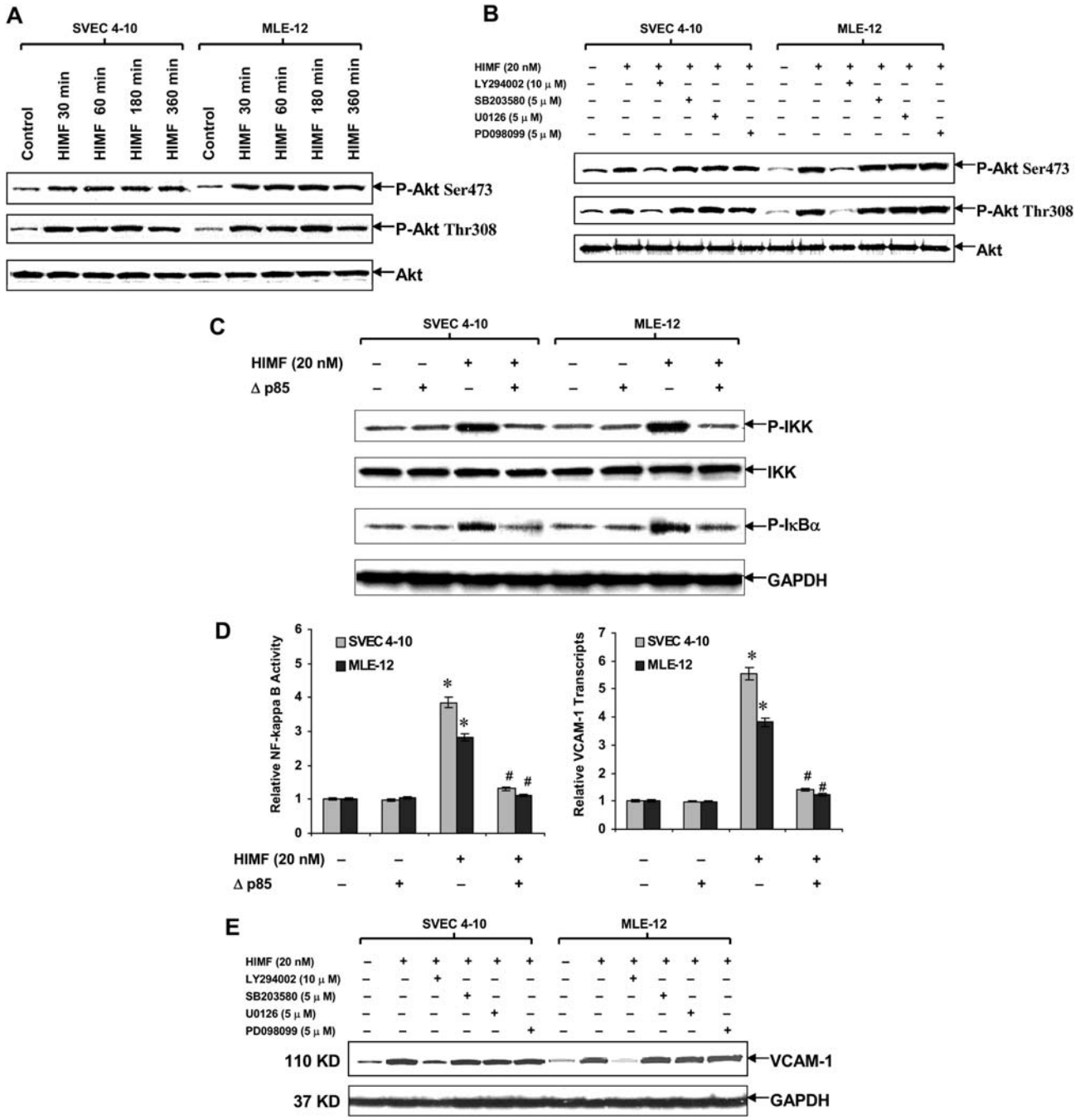


*Figure 8.* PI-3K/Akt pathway is involved in HIMF-induced NF-κB activation and VCAM-1 production. SVEC 4-10 and MLE-12 were pretreated with signal transduction inhibitors, or cotransfection of luciferase constructs and dominant-negative mutants, and stimulated with HIMF protein (20 nmol/liter) for various periods, as indicated. (*A*) HIMF strongly induced the phosphorylation of Akt at Ser473 and Thr308. The Akt activation (phosphorylation) started at 30 min and was sustained for 360 min. (*B*) PI-3K inhibitor, LY294002 (10 μmol/liter), abolished HIMF-induced Akt phosphorylation. However, SB203580 (5 μmol/liter), PD098059 (5 μmol/liter), or U0126 (5 μmol/liter) did not block HIMF-induced Akt phosphorylation at 180 min. (C) Transfection of Δp85, a dominant-negative mutant of PI-3K, into SVEC 4-10 and MLE-12 cells abolished HIMF-induced phosphorylation of IKK and IκBα at 180 min. (*D*) Transfection of Δp85 into SVEC 4-10 and MLE-12 cells also blocked HIMF-induced NF-κB activation and VCAM-1 transcription activity at 24 h. (*E*) LY294002 (10 μmol/liter), but not SB203580 (5 μmol/liter), PD098059 (5 μmol/liter), or U0126 (5 μmol/liter), specifically suppressed HIMF-induced VCAM-1 production in SVEC 4-10 and MLE-12 cells at 24 h. *Asterisks* indicate a significant increase from SVEC 4-10 or MLE-12 controls treated without HIMF ($P < 0.05$). *Pound symbols* indicate a significant decrease from SVEC 4-10 or MLE-12 cells treated with HIMF only ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

production. These results placed PI-3K/Akt downstream of HIMF, but upstream of NF-κB.

In summary, the present study shows that HIMF induces VCAM-1 production in mouse lung tissues, cultured endothelial cell lines, and epithelial cell lines. The induction is dependent on activation of NF-κB, an important transcription factor that binds VCAM-1 promoter. We also explored how NF-κB is activated, and found that PI-3K/Akt pathway acts upstream of IKK

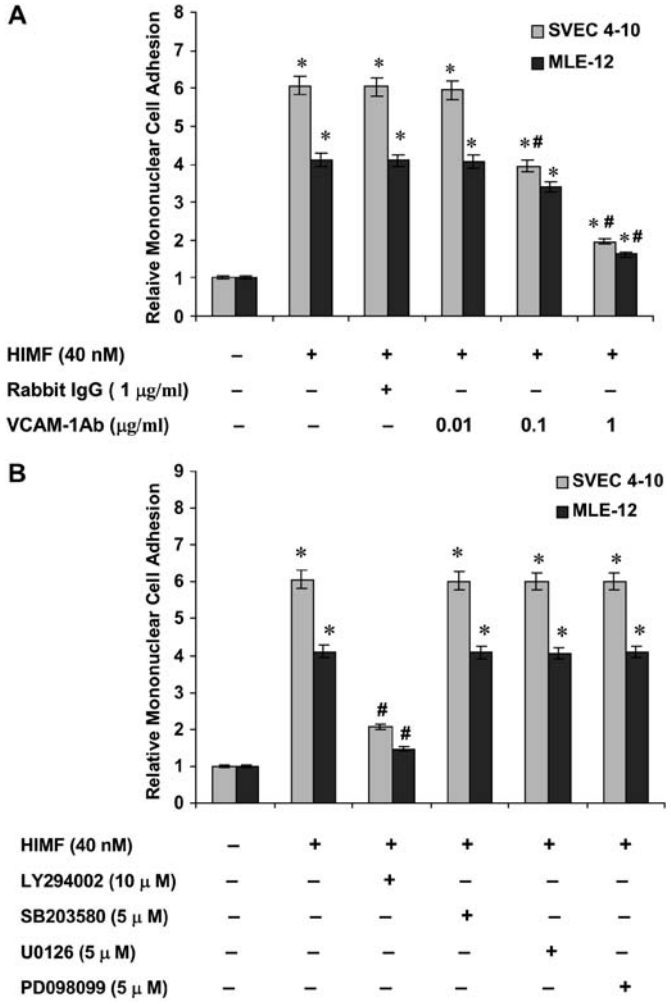


***Figure 9.*** HIMF-induced VCAM-1 upregulation promotes mononuclear cell adherence. Mononuclear cells were isolated from heparinized mouse blood using Ficoll-Paque, labeled with a fluorescein-labeled antibody against CD45, and incubated with SVEC 4-10 and MLE-12 cells for 30 min at 37°C in 5% $CO_2$. The nonadherent cells were removed by carefully washing the cells twice with medium. The number of adherent mononuclear cells was determined by counting five random high-power fields. (*A*) Incubation of SVEC 4-10 and MLE-12 cells with HIMF (40 nmol/liter) protein for 24 h resulted in an increase of mononuclear cell adherence to them, which was blocked by anti–VCAM-1 antibody, but not by rabbit IgG. (*B*) When SVEC 4-10 and MLE-12 cells were pretreated with PI-3K inhibitor, LY294002 (10 μmol/liter), 1 h before HIMF (40 nmol/liter) incubation, the number of adherent mononuclear cells was significantly decreased. However, SB203580 (5 μmol/liter), PD098059 (5 μmol/liter), or U0126 (5 μmol/liter) did not abolish HIMF-induced mononuclear cell adherence to SVEC 4-10 and MLE-12 cells. *Asterisks* indicate a significant increase from SVEC 4-10 or MLE-12 controls treated without HIMF ($P < 0.05$). *Pound symbols* indicate a significant decrease from SVEC 4-10 or MLE-12 cells treated with HIMF only ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

signalsome. Combined with our recent findings that LPS induces HIMF production in mouse lungs, these results suggest that HIMF plays critical roles in lung inflammatory processes, especially inflammatory cell adhesion and migration.

***Conflict of Interest Statement***: None of the authors has a financial relationship in a commercial entity that has an interest in the subject of this manuscript.

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