The Adaptor Molecule Lnk Negatively Regulates Tumor Necrosis Factor-α-dependent VCAM-1 Expression in Endothelial Cells through Inhibition of the ERK1 and -2 Pathways*

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Lnk, with APS and SH2-B (Src homology 2-B), belongs to a family of SH2-containing proteins with potential adaptor functions. Lnk regulates growth factor and cytokine receptor-mediated pathways implicated in lymphoid, myeloid, and platelet homeostasis. We have previously shown that Lnk is expressed and up-regulated in vascular endothelial cells (ECs) in response to tumor necrosis factor-α (TNFα). In this study, we have shown that, in ECs, Lnk down-regulates the expression, at both mRNA and protein levels, of the proinflammatory molecules VCAM-1 and E-selectin induced by TNFα. Mechanistically, our data indicated that, in response to TNFα, NFκB/p65 phosphorylation and translocation as well as IκBα phosphorylation and degradation were unchanged, suggesting that Lnk does not modulate NFκB activity. However, Lnk activates phosphatidylinositol 3-kinase (PI3K) as reflected by Akt phosphorylation. Our results identify endothelial nitric-oxide synthase as a downstream target of Lnk-mediated activation of the PI3K/Akt pathway and HO-1 as a new substrate of Akt. We found that sustained Lnk-mediated activation of PI3K in TNFα-activated ECs correlated with the inhibition of ERK1/2 phosphorylation, whereas phosphorylation of p38 and c-Jun NH2-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) was unchanged. ERK1/2 inhibition decreases VCAM-1 expression in TNFα-activated ECs. Collectively, our results identify the adaptor Lnk as a negative regulator in the TNFα-signaling pathway mediating ERK inhibition and suggest a role for Lnk in the interplay between PI3K and ERK triggered by TNFα in ECs.

In their normally quiescent state, endothelial cells (ECs)4 maintain blood flow, allowing the continuous traffic of plasma and cellular constituents between blood and tissues. To accomplish this function, ECs must control vascular tone and leukocyte adhesion as well as coagulation and thrombosis. However, when ECs are exposed to proinflammatory stimuli, they become activated and promote vasoconstriction and leukocyte adhesion and activation, as well as coagulation and thrombosis (1, 2). These functional changes are due to the expression by activated ECs of a series of proinflammatory genes encoding adhesion molecules, cytokines/chemokines, and costimulatory and procoagulant molecules. EC activation and subsequent injury is a prominent feature associated with acute and/or chronic inflammation such as occurs during hyperoxia (3), endotoxic shock (4), arteriosclerosis (5), ischemia-reperfusion injury (6), and acute or chronic graft rejection (7).

Critically, cytokine-treated ECs express de novo a set of cell adhesion molecules, namely E-selectin, and integrin ligands, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which can interact with blood leukocytes (1, 2, 8). Cytokine-activated ECs also secrete and display chemokines, which stimulate leukocyte chemokinesis (9). The leukocyte integrins LFA-1 and VLA-4 are the counterreceptors for ICAM-1 and VCAM-1 on ECs, and enhanced interactions produce firm attachment to the ECs. A central role of TNF in inflammation has been established by the elucidation of signaling pathways mediated by TNF in ECs (10). Tumor necrosis factor signaling in ECs involves several pathways, including the NFκB, the phosphatidylinositol 3-kinase (PI3K), and members of the MAP3K (mitogen-activated protein kinase kinase kinase) family pathways. Signaling is initiated when ligand-occupied TNF receptors recruit the binding of intracellular adaptor proteins TRADD (TNF receptor-associated death domain protein), RIP (receptor-interacting protein, and TRAF2 (TNF receptor-associated factor 2). The physiological downstream targets of RIP and TRAF2 remain uncertain. Moreover, potential cross-talk between TNF-induced signaling pathways (i.e. NFκB, PI3K, MAPKs) is not well established.

Lnk, with the closely related proteins APS and Src homology 2-B (SH2-B), belongs to a subfamily of SH2-containing proteins.
with potential adaptor functions (11–13). Substantial data suggest that these adaptor proteins are regulators of growth factor and cytokine receptor-mediated pathways. Lnk protein contains an NH₂-terminal proline-rich region, a pleckstrin homology domain, an SH2 domain, and potential tyrosine phosphorylation sites. Lnk−/− mice display an abnormal accumulation of erythroid cells, megakaryocytes, and B lymphocytes in the different hematopoietic compartments, indicating a defect in lymphoid and myeloid homeostasis (14). Lnk negatively regulates stem cell factor c-kit/stem cell factor signaling in B cell precursors, hematopoietic progenitor cells, and mast cells (15–18).

We have previously shown (19) that Lnk is expressed and up-regulated in vascular ECs in response to TNFα. We hypothesized that the adaptor Lnk may be implicated in the TNFα-signaling cascade. In this study, we showed that Lnk is a negative regulatory component of TNF signaling in human ECs, modulating both PI3K and MAPK ERK1/2 activity.

MATERIALS AND METHODS

Reagents—Recombinant human TNFα was provided by Prof. P. Neuman, (BASF, Ludwigshafen, Germany). LY294002 (10 μM), wortmannin (100 nM), SB203580 (10 μM), PD98059 (50 μM), and SP600125 (10 μM) inhibitors were purchased from Sigma. Tin protoporphyrin IX (SnPP) (10 μM) was purchased from Frontier Scientific (Logan, UT).

Goat polyclonal anti-Lnk antibody was obtained from Sero-tec (Cergy St. Christophe, France) (diluted 1/500). TNF-receptor 1 expression was measured with a mouse anti-TNF-receptor 1 antibody from BD Biosciences. Mouse monoclonal antibodies directed against human VCAM-1 (clone E-10) were purchased from Santa Cruz Biotechnology, Inc. for flow cytometry analysis (1:1000 dilution) and from R & D Systems (Lille, France) for Western blot analysis (1:100 dilution). Mouse E-selectin antibody was purchased from R & D Systems (1:1000 and 1:100 dilution). Rabbit polyclonal and mouse polyclonal antibodies directed against total and phosphorylated forms of Akt (at Ser-473), IκBα (at Ser-32), NFκB p65 (at Ser-536), NFκB p65 total, eNOS (at Ser-1177), total eNOS, p38 (at Thr-180/Tyr-182), ERK1/2 p42/p44 (at Thr-202/Tyr-204) and JNK (at Thr-183/Tyr-185) were purchased from Cell Signaling Technology (Ozyme, St. Quentin Yveline, France) (1:1000 dilution). Mouse anti-human Lnk was a kind gift from Dr. Jun Hayashi (School of Medicine, University of Maryland, Baltimore, MD). The 2.0-kbp SnaBI-Xbal fragment from pcDNA3hLnk, containing the entire coding region and a 1.415-kbp fragment containing an internal ribosome entry site (IRES) followed by cDNA encoding green fluorescent protein (GFP) under the cytomegalovirus promoter from the pT/BH plasmid were cloned into the vector pShuttleCMV. The expression cassette contains a single promoter, which, with the IRES, permits the translation of two open reading frames from one mRNA transcript. The resultant pShuttleCMV-Lnk-IRES-eGFP vector was tested in HUVEC transfections before adenovirus generation.

The recombinant adenovirus AdLnk was produced in the human embryonic kidney 293 cells by the vector core laboratory of the University of Nantes (INSERM ERM-0105 Gene Therapy Laboratory, Nantes, France) as previously described (21). The recombinant adenovirus AdTrack GFP was used as a control (AdGFP). HUVECs were cultured in six-well plates at 70% confluence and infected with a multiplicity of infection of 500 infectious particles/cell for AdLnk and 10 infectious particles/cell for AdTrack GFP. Adenoviral infection was carried out in endothelial cell growth medium supplemented with 1% FCS and penicillin-streptomycin at 37 °C, 5% CO₂ after infection. The cells were washed with medium containing 10% FCS and grown in fresh supplemented endothelial cell growth medium. The expressions of GFP and Lnk were measured 24 h after infection.

Transfections and Promoter-Reporter Assays—ECs were transfected 12 h post-infection with AdGFP or AdLnk. The transfections were performed using Lipofectamine+ reagent (Invitrogen) and a VCAM-1 promoter-luciferase reporter construct kindly provided by Dr. M. Soares (Institut Gulbenkian de Ciência, Oeiras, Portugal). The transfected cells were plated in 24-well plates and incubated with TNFα (100 units/ml) for various periods of time. Untreated and TNFα-treated control ECs were used as negative and positive controls, respectively. Cell extracts were obtained using the Promega (Madison, WI) cell culture lysis reagent extraction kit, and luciferase activity was assayed (luciferase assay system, Promega) according to the manufacturer’s instructions. Results shown are the mean ± S.E. from four separate experiments performed in duplicate wells and are expressed in arbitrary luciferase units.

Flow Cytometry and Immunofluorescence—After treatment, HUVECs were harvested using trypsin/EDTA (100 μl/well; 1%
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**FIGURE 1.** TNFα induces the transient expression of Lnk in human ECs. Confluent EC monolayers were incubated with 100 units/ml TNFα for the indicated periods. Cells were lysed in parallel experiments to allow RNA and protein analysis. A, semiquantitative RT-PCR and southern blotting of PCR products for Lnk, VCAM-1, and β-actin. B, real time quantitative RT-PCR for Lnk in TNFα-activated ECs. Results shown are the mean ± S.E. from three independent experiments and are expressed as relative expression calculated according to the 2−ΔΔCt method after normalization to β-actin levels (*, p < 0.05 versus control). C, a representative analysis of Lnk protein expression by Western blotting. Blots were reprobed with an anti-tubulin antibody to ensure equal loading. Relative Lnk quantification by densitometry after Western blotting from three independent experiments is shown below the blots. Data are the mean ± S.E. expressed as arbitrary units (A.U.) after sample normalization to tubulin level (*, p < 0.05 versus control). D, a representative analysis showing Lnk tyrosine phosphorylation and cytoplasmic expression after a short time of stimulation with TNFα (100 units/ml). Immunoprecipitations were performed using anti-Lnk antibodies and Western blotted using anti-phosphotyrosine monoclonal antibodies (upper panel) and anti-Lnk antibodies (lower panel).

in PBS) and washed in PBS, 1% FCS, and 0.1% NaNO3 at 4 °C. The cells were incubated with anti-human E-selectin, VCAM-1, ICAM-1, or TNF-receptor 1 monoclonal antibodies (10 μg/ml in PBS, 1% FCS, 0.1% NaN3) for 30 min at 4 °C. After washing in PBS, 1% FCS, 0.1% NaNO3, the cells were stained (30 min, 4 °C) with a phycoerythrin-labeled polyclonal goat anti-mouse IgG F(ab')2 antibody (Jackson Laboratories, West Grove, PA). Control staining was with irrelevant isotype-matched antibodies. Fluorescent labeling was measured on 10,000 cells/sample using a FACsCalibur® fluorescence-activated cell sorter and analyzed with CellQuest® software (BD Biosciences).

For immunofluorescence, ECs were grown to confluence on glass coverslips. After treatment, the cultures were washed with PBS, fixed for 20 min in PBS containing 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 15 min. The cells were washed again with PBS, incubated overnight at 4 °C with blocking buffer (5% bovine serum albumin in PBS), and then incubated with monoclonal anti-p65 antibodies (10 μg/ml) for 4 h. The slides were re-washed and incubated with phycoerythrin-conjugated goat anti-mouse antibodies (5 μg/ml, Jackson Laboratories) for 1 h. The slides were washed in PBS, dried, and mounted with ProLong® anti-fade reagent (Molecular Probes). Specimens were examined by immunofluorescence microscopy using a Nikon Diaphot microscope.

**Cell Lysis, Immunoprecipitation, and Western Blot Analysis**—Cell lysis was performed on ice in 100 mM NaCl, 5 mM MgCl2, and 1% Nonidet P-40 in the presence of the protease inhibitors AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (protease inhibitor mixture; Sigma). Protein concentration was determined using BCA protein assay reagent (Pierce). Immunoprecipitation assays were performed using protein G-Sepharose 4 Fast Flow (immunoprecipitation starter kit, Amersham Biosciences). Cell lysates were incubated overnight at 4 °C with protein G and 10 μg/ml of anti-Lnk or anti-P-Tyr antibodies. Cell lysates (20 μg) and immunoprecipitation were resolved by SDS-PAGE (7.5–15%), and proteins were transferred to nitrocellulose membranes (ECL Hybond™, Amersham Biosciences) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Thereafter, membranes were subjected to Western immunoblot analysis using the primary antibodies described above and appropriate peroxidase-conjugated secondary antibodies. Antibody-bound proteins were detected using an enhanced chemiluminescence ECL™ Western blotting analysis system (Amersham Biosciences), and the membranes were exposed to Kodak X-Omat LS x-ray film. Blot quantification was performed with a Kodak Digital Science Image Station 440 CF and Kodak Digital Science Image Analysis 1D® software.

**Cellular DNA Content Analysis**—Cellular DNA content analyses were performed by flow cytometry as follows. After treatment, ECs were harvested using trypsin, washed twice in PBS, fixed in ice-cold 70% ethanol, and incubated for at least 24 h at 4 °C. Fixed cells were then stained with 50 μg/ml pro-pidium iodide, 100 units/ml RNase A, 1 mg/ml glucose in PBS (1 ml/1 × 10⁶ cells). Fluorescence was measured on 10,000 cells/sample using a FACsCalibur® (BD Biosciences) and analyzed using CellQuest® software (BD Biosciences). Results shown are representative of at least three independent experiments.

**Semi-quantitative Reverse Transcription (RT)-PCR and Southern Blotting**—RNA from treated ECs was collected by TRIzol extraction (Invitrogen). Genomic DNA was removed by DNase treatment (Roche Diagnostics), and mRNAs were reverse-transcribed into first strand cDNAs using poly(dT) oli-
ggonucleotide and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The following oligonucleotides were used in this study as primers in PCR and to design probes: human Lnk forward, 5'-CCAGGAGAACCTTGTTG-3' and reverse, 5'-GGCAGAATCTGCTTCTTGAGG-3'; human VCAM-1 forward, 5'-CTCATTATGCTATG-3' and reverse, 5'-GATTCTACCTTCTAAGAC-3'; human β actin forward, 5'-AATCCTGGACACACCTTCTACA-3' and reverse, 5'-GCACGTAGACCGCTTCTCCTTA-3' (Genosys, Sigma). Target transcripts were amplified in 25 μl of PCR mix containing 10× buffer, 50 mM MgCl₂, dATP, dCTP, dGTP, and dTTP all at 2.5 mM, 100 pmol/μl each primer, and TaqDNA polymerase (Invitrogen).

The cycling conditions were 5 min at 95 °C to activate the TaqDNA polymerase, cycles of 1 min at 95 °C and 45 s at both 60 and 72 °C (between 18 and 23 cycles were used depending on the primers), and the reaction finished with 3 min at 72 °C and storage at 4 °C. Amplicons were then separated on 1% agarose gels containing ethidium bromide and transferred onto Hybond N+ membrane (Amer sham Biosciences) overnight. Hybridizations were performed at 45 °C in prehybridization/hybridization solution (5 × SSC, 5 × Denhart’s solution, 0.5% SDS, and denatured herring sperm). Probes were labeled with [32P]dCTP using the Rediprime DNA labeling system (Amer sham Biosciences). Autoradiographs were analyzed with IPLabel for quantification and Kodak Digital Science Image Station 440 CF and Kodak Digital Science Image Analysis 1D® software for image analysis.

Quantitative Real Time PCR—Real time quantitative PCR was performed in an Applied Biosystems GenAmp 7700 sequence detection system using labeled TaqMan® probes (Applied Biosystems, Foster City, CA). The following oligonucleotide sets purchased from Applied Biosystems were used: VCAM-1 (Hs00365486_m1), HO-1 (Hs00157965_m1), and (Hs00193878_m1). Hypoxanthine guanine phosphoribosyl transferase was used as an endogenous control to normalize RNA amount. Relative expression between a given sample and a reference sample was calculated according to the 2^ΔΔCt method (PE Applied Biosystems, Foster City, CA), where the reference represents one-fold expression, as previously described (22).

Statistical Analysis—Results are expressed as means ± S.E. Statistical analysis was performed using two-tailed Student’s t tests and analysis of variance when more than two treatment regimens were compared. A value of p < 0.05 was considered statistically significant.

RESULTS

We previously reported up-regulation of Lnk mRNA in porcine ECs treated with recombinant TNFα (19). In this study, using cultured ECs, we showed that recombinant human TNFα also induced human Lnk expression at the mRNA level in human ECs. Induction of VCAM-1 was used as a control to assess TNFα-mediated EC activation. Induction of Lnk transcripts, investigated by both semiquantitative RT-PCR (Fig. 1A) and quantified by real time RT-PCR (Fig. 1B) was transient and maximal after 2 h of incubation. Moreover, concomitant with the increase in mRNA, a 2.8-fold increase in Lnk protein level, as assessed by Western blotting, was observed at 2 h and declined after 4 h of treatment with TNFα (Fig. 1C). Immunoprecipitation assays showed an early and transient tyrosine phosphorylation of Lnk associated with an increase in the cytoplasmic Lnk level, which both peak 15 min after stimulation with TNFα, thus preceding mRNA and protein regulation (Fig. 1D). These data indicate that TNFα specifically regulates the expression of the adaptor protein Lnk at both mRNA and protein levels probably as an autoregulatory loop resulting from early Lnk
phosphorylation and activation. These findings suggest that, in EC treated with TNFα, the adaptor molecule Lnk may be involved in early signaling events (within 15–30 min) concomitant to NFκB, PI3K, and MAPK activation.

To investigate the role of Lnk in EC activation, we first generated a recombinant adenoviral vector encoding human Lnk and GFP as a reporter gene (AdLnk) to mimic the transient overexpression of Lnk mediated by TNFα. This vector contains a single promoter, which with the IRES, permits the translation of two proteins from one mRNA transcript. Controls were nontransduced cells and cells transduced with a recombinant adenovirus encoding GFP alone (AdGFP) to account for any effects that may be due to adenoviral infection. Expression of GFP and Lnk in cultured HUVECs was examined 24 h post-infection. Adenoviral vectors allowed the transduction of 90–95% of ECs for both vectors, as reflected by GFP expression (Fig. 2A). Fig. 2B shows the dose-dependent induction of Lnk protein in transduced ECs assessed by Western blotting using anti-Lnk-specific antibodies. We did not observe any apparent toxic effects of Lnk, as judged by propidium iodide staining and subsequent viability and apoptosis analysis by flow cytometry (Fig. 2C).

According to the previously identified functions of Lnk in other cell types, we hypothesized that Lnk may either promote or inhibit EC activation (14, 16, 23, 24). Therefore, we first sought to determine whether Lnk expression was able to induce E-selectin and VCAM-1 expression on quiescent ECs, a typical feature of EC activation in response to proinflammatory cytokines, including TNFα and interleukin-1β (2, 10). HUVECs were transduced with AdLnk or AdGFP for 24 h, and the cell surface expression of VCAM-1, E-selectin, and ICAM-1 was examined by flow cytometry of GFP-positive transduced ECs. Quiescent, nontransduced HUVECs do not express VCAM-1 and E-selectin but do express a basal level of ICAM-1. We found that Lnk expression was not associated with the induction of E-selectin or VCAM-1 in transduced ECs, suggesting that Lnk does not promote EC activation (Fig. 2D).

Next, we examined the effect of Lnk on TNFα-induced EC activation. HUVECs were transduced with AdLnk or AdGFP for 24 h, and the cell surface expression of VCAM-1, E-selectin, and ICAM-1 was examined by flow cytometry of GFP-positive transduced ECs. Quiescent, nontransduced HUVECs do not express VCAM-1 and E-selectin but do express a basal level of ICAM-1. We found that Lnk expression was not associated with the induction of E-selectin or VCAM-1 in transduced ECs, suggesting that Lnk does not promote EC activation (Fig. 2D).

**FIGURE 3.** Lnk decreases VCAM-1 expression caused by TNFα in ECs. A, HUVECs were noninfected (NI) or transduced with either control (AdGFP) or AdLnk, and TNFα (100 units/ml) was added for 6 h. Immunostaining was performed using specific anti-human E-selectin, VCAM-1, or ICAM-1 monoclonal antibodies (bold lines) or irrelevant isotype-matched control monoclonal antibodies (thin lines). Antibody binding was detected using phycoerythrin-labeled anti-mouse IgG and analyzed using a FACSCalibur®. Data are presented as histograms plotting mean fluorescence intensity on a four-decade logarithmic scale (x-axis) versus cell number (y-axis). Mean fluorescence intensities are indicated. The right panel shows VCAM-1 expression on the cell surface, expressed as mean fluorescence intensities, determined from five independent experiments (*, \( p < 0.05 \) versus noninfected (NI); **, \( p < 0.05 \) versus ECs transduced with AdGFP). B, HUVECs were transduced with either control or AdLnk, and TNFα (100 units/ml) was added for 6 h. VCAM-1 expression was determined by immunoblotting. Equal protein loading was confirmed using an anti-tubulin antibody. Data shown are representative of three independent experiments. The right panel shows total VCAM-1 protein level expressed as a percentage of expression relative to controls from three independent experiments (*, \( p < 0.05 \) versus NI; **, \( p < 0.05 \) versus ECs transduced with AdGFP; \( n = 3 \)). C, Lnk down-regulates VCAM-1 mRNA expression caused by TNFα. ECs were either noninfected or infected with AdGFP as a control or AdLnk. Total RNA was prepared 6 h after TNFα exposure. Quantification of mRNA for VCAM-1 was performed by real time RT-PCR, and results were expressed as relative expression calculated according to the \( 2^{-\Delta\Delta C_t} \) method, after normalization to hypoxanthine guanine phosphoribosyltransferase levels (*, \( p < 0.05 \) versus NI; **, \( p < 0.05 \) versus ECs transduced with AdGFP). Results are representative of at least three separate experiments. D, VCAM-1 transcription activity in EC transiently transfected with a VCAM-1 promoter/luciferase reporter DNA construct. HUVECs were noninfected (NI) or transduced with either control (AdGFP) or AdLnk 3 h before transfection. After transfection, TNFα (100 units/ml) was added for 0, 2, 6, and 8 h. Results shown are the mean ± S.E. from four separate experiments performed in duplicate wells and are expressed in arbitrary luciferase units (A.U.) (*, \( p < 0.05 \) versus NI; **, \( p < 0.05 \) versus ECs transduced with AdGFP).
for 18 h. The cells were then stimulated with vehicle (culture medium) or TNFα (100 units/ml) for 6 h. Cell surface expression of VCAM-1, E-selectin, and ICAM-1 was then examined by flow cytometry of GFP-positive transduced ECs. As shown in Fig. 3A, TNFα treatment for 6 h alone stimulated ICAM-1, VCAM-1, and E-selectin expression significantly. Compared with TNFα treatment alone for 6 h, Lnk expression significantly reduced TNFα-mediated expression of VCAM-1 and E-selectin at the cell surface (Fig. 3A). In contrast, ICAM-1 up-regulation by TNFα was unaffected (Fig. 3A), suggesting a selective effect of Lnk on TNFα signaling. The global decrease in the cell surface expression of VCAM-1 on Lnk-expressing ECs, expressed as mean fluorescence intensity, was 67.4 and 78.8% versus nontransduced (*, p < 0.05) and AdGFP-transduced controls (**, p < 0.05), respectively (Fig. 3A). The decrease in VCAM-1 expression correlated with GFP expression in AdLnk-transduced ECs and was consistently observed at 2, 6, and 8 h after stimulation (data not shown). Lnk-mediated inhibition of total VCAM-1 protein level, measured by Western blotting, was 47.6% ± 5.8% compared with controls (*, p < 0.05) (Fig. 3B). Cell surface expression of TNF receptor-1 (p55) was not modified in transduced cells and thus could not account for the inhibitory effect observed in Lnk-expressing ECs (data not shown). To determine whether the negative regulatory effect of Lnk on VCAM-1 expression results from a decrease in VCAM-1 gene transcription, quantitative RT-PCR was performed on controls and AdLnk-transducedHUVECs. Cells expressing Lnk had reduced levels of VCAM-1 mRNA (Fig. 3C), suggesting that Lnk-mediated regulation may occur at the transcription level. We monitored VCAM-1 transcription activity in ECs transiently transfected with a VCAM-1 promoter/luciferase reporter plasmid. In ECs exposed to TNFα, VCAM-1 activity increased by 18-fold at 8 h compared with controls not exposed to TNFα. Overexpression of Lnk inhibited TNFα-driven VCAM-1 luciferase activity by 50% compared with control ECs (Fig. 3D).

In ECs, TNFα activates three major signaling pathways implicated in inflammatory gene expression and cytoprotective processes: the NFκB, PI3K, and MAPK pathways (10, 25, 26). To determine the effects of Lnk on NFκB, PI3K, and MAPK (JNK, ERK1/2, and p38) activities, normal and Ad-transduced HUVECs were exposed to 100 units/ml TNFα. First, NFκB activity was evaluated in cytosolic fractions by Western blotting analysis of the phosphorylation and degradation of IκBα and the phosphorylation and translocation of NFκB/p65 (Fig. 4A). On stimulation of ECs with TNFα, rapid degradation of IκBα, preceded by its phosphorylation, was observed within 15 min. Western blot analysis revealed no effect on either the TNFα-induced phosphorylation (Fig. 4A, upper panels) or degradation (lower panels) of IκBα, but Lnk seemed to delay the reappearance of IκBα. Similarly, no significant change was observed on NFκB/p65 phosphorylation (Fig. 4A, upper panels) and translocation to the nucleus (lower panels) in Lnk-expressing ECs. Failure of Lnk to impair p65 translocation was further confirmed by immunoﬂuorescence (Fig. 4B). Therefore, Lnk seems to have no significant effect on NFκB activity.

In nontransduced ECs, TNFα also rapidly increased JNK, ERK1/2, and p38 activity in HUVECs (Fig. 5), as reported by others (27). JNK phosphorylation peaked at 10 min (8.5 ± 0.8-fold) and returned to base line by 60 min. p38 activation in response to TNFα was similar to JNK, with a peak at 10 min (6.1 ± 1.1-fold). Overexpression of Lnk did not appear to decrease TNFα activation of JNK or p38 MAPK (Fig. 5). In contrast, Lnk was found to block almost completely ERK1/2 phosphorylation mediated by TNFα in ECs. ERK1/2 phosphorylation by TNFα was similar in nontransduced ECs and ECs transduced with control AdGFP, with a maximal increase at 10 min (18.5 ± 3.9-fold and 24.6 ± 6.1-fold for ERK2, respec-
tively) and a return to base line at 30 min (Fig. 5). In contrast, phosphorylation of ERK1/2 was totally abrogated in Lnk-expressing ECs, suggesting that Lnk inhibits activation of ERK1/2. Together, these results suggest that Lnk selectively prevents activation of the ERK pathway without affecting the JNK and p38 pathways. However, high levels of phosphorylated Akt were found in untreated (0 min) ECs expressing Lnk and remained significantly elevated upon stimulation with TNFα, whereas total Akt levels remained unchanged (Fig. 5). Together, these results suggest that Lnk triggers signaling events affecting both the PI3K and the ERK1/2 pathways.

Next, to determine whether Lnk was able to mediate signaling events in the absence of TNF stimulation, the phosphorylation state of Lnk in transduced ECs was analyzed after immunoprecipitation with anti-phosphotyrosine and anti-Lnk antibodies. Our data show that Lnk becomes tyrosine-phosphorylated in HUVECs transduced with AdLnk but not with AdGFP (Fig. 6A). To evaluate whether Lnk activates signaling pathways, HUVECs were exposed to either AdGFP or AdLnk. Western blot analysis was performed 24 h after transduction. We first examined the effect of Lnk on the PI3K pathway, which was found to be persistently activated in response to TNF (Fig. 6B). Akt (protein kinase B) is well described as a mediator of EC survival and proliferation through activation of various downstream molecular targets (28). Concurrent with Lnk expression, we also found strong induction of HO-1 protein (Fig. 6C). In addition, cells expressing Lnk were found to contain elevated levels of both eNOS and phosphorylated eNOS (Fig. 6C). Lnk-mediated HO-1 expression at the protein level was paralleled at the mRNA level (data not shown).

To determine whether the regulation of HO-1 and eNOS results from activation of the PI3K pathway, ECs were transduced with AdLnk or AdGFP in the presence or absence of LY294002, a PI3K inhibitor, and the expression of HO-1 and phosphorylated eNOS were assayed by Western blotting. PI3K inhibition was validated by analysis showing that, under our conditions, LY294002 efficiently prevents Akt phosphorylation (Fig. 6C). The induction of HO-1 by Lnk was also totally inhibited by LY294002, whereas eNOS phosphorylation was strongly inhibited compared with controls. These results indicate that, in ECs, Lnk induces HO-1 and promotes eNOS activity through a PI3K/Akt-dependent mechanism.

We further explored the involvement of Lnk in potential cross-talk between the ERK and the PI3K pathways. Thus, we investigated the ability of the PI3K inhibitor LY294002 to block TNF-induced ERK1/2 phosphorylation in ECs and, conversely, the ability of the MEK1 inhibitor PD98059 to block TNF-induced Akt phosphorylation. The data shown in Fig. 7 indicate that, in nontransduced HUVECs treated with TNFα, inhibition of PI3K using LY294002 strongly increases ERK1/2 activity through its phosphorylation (Fig. 7A), as previously reported (26). Moreover, we found that inhibition of ERK1/2 using PD98059 activates the PI3K pathway, as reflected by an increase
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**FIGURE 7.** The inhibitory effect of Lnk on ERK signaling is PI3K-independent. HUVECs were incubated with or without TNFα (100 units/ml) for 20 min. A, pretreatment with the PI3K inhibitor LY294002 (10 μM) was performed for 3 h before cytokine treatment. Cells were lysed, and cytosolic proteins were electrophoresed and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. B, pretreatment with PD98059 (50 μM) was performed for 3 h before cytokine treatment. C, pretreatment with wortmannin (100 nM) was performed for 1 h prior to activation with TNFα. Cytoplasmic proteins from equal numbers of cells were subjected to Western blotting analysis. ERK activation was determined by immunoblotting using phosphospecific (P-) antibodies. Variations in total ERK levels reflect ERK1/2 translocation to the nucleus. Blots were reprobed with anti-tubulin monoclonal antibody. Representative blots from three independent experiments are shown.

in Akt phosphorylation (Fig. 7B). Together, these data demonstrate that there is an interplay between the ERK and PI3K pathways in TNFα-activated ECs by confirming the negative regulatory control of PI3K on ERK activation and revealing a reverse negative regulatory control of ERK on PI3K activation. We speculate that activation of the PI3K-signaling cascade by Lnk could subsequently inhibit ERK activity in ECs. To address this question, nontransduced HUVECs (noninfected) and HUVECs transduced with AdLnk were pretreated with PI3K inhibitors (wortmannin and LY294002) for 1 h before incubation with TNFα. As shown in Fig. 7C, both inhibitors caused an increase in ERK1/2 phosphorylation in nontransduced ECs. This induction of phosphorylation was inhibited in ECs expressing Lnk. These data show the ability of Lnk to inhibit ERK1/2 activation independently of PI3K.

Finally, we asked whether Lnk-mediated VCAM-1 down-regulation results from activation of PI3K and its downstream targets eNOS and HO-1 or from inhibition of ERK1/2 activity. Thus, we investigated the activity of the ERK and PI3K inhibitors to block TNF-induced VCAM-1 expression in ECs (Fig. 8A). Our results clearly demonstrate that the PI3K inhibitor wortmannin and inhibitors of NO (l-nitro arginine methyl ester) and HO-1 (SnPP) have no effect on VCAM-1 expression. In contrast, the ERK inhibitor PD98059 efficiently blocks TNF-induced VCAM-1 expression in nontransduced ECs (4.7 ± 2.0 versus 21.5 ± 5.8, with and without inhibitor, respectively; *, p < 0.05) and potentiates the inhibitory effect of Lnk in transduced ECs, whereas inhibitors of p38 (SB203580) and JNK (SP600125) have no effect. Inhibition of VCAM-1 was confirmed by Western blotting (Fig. 8B). It is notable that, in our experimental conditions, blocking PI3K with LY294002 significantly decreased VCAM-1 expression, whereas wortmannin had no significant effect. This discrepancy may reflect the recently reported divergent actions of these inhibitors (29).

Collectively, our findings suggest that in ECs, Lnk controls two major pathways implicated in TNF signaling, the PI3K and the ERK pathways. Our study identifies a regulatory role for Lnk on VCAM-1 expression mediated via inhibition of the ERK MAPK.

**DISCUSSION**

This study provides evidence that the adaptor protein Lnk acts as a regulatory mediator modulating the proinflammatory action of TNFα on vascular ECs in vitro. In particular, Lnk could counteract the increase in leukocyte adhesion to endothelial cells attributable to TNFα, which is crucial to the inflammatory reaction. To our knowledge, these results are the first demonstration of a physiologic function of the adaptor Lnk in vascular endothelium. This anti-inflammatory effect of Lnk is correlated with the inhibition of the induction of VCAM-1 in response to TNFα. Our data also shows that Lnk slightly reduces TNFα-induced E-selectin expression. The effect of Lnk on E-selectin regulation was weaker than that observed on VCAM-1.

Previous studies have shown that TNFα up-regulates a number of genes, including prothrombotic factors, chemokines, and cellular adhesion molecules, many of which depend on the action of NFκB, which is thought to play a central role in the regulation of inflammatory mediators (10, 25). Our results show that Lnk does not inhibit phosphorylation and subsequent degradation of IκBα. Similarly, phosphorylation and translocation of NFκB p65 were unmodified. These findings suggest that the negative regulatory effect of Lnk is not due to the suppression of endothelial NFκB activation mediated by TNFα. However, we cannot rule out the possibility that Lnk acts on another step of the NFκB pathway leading to modulation of NFκB transcriptional activity.

In ECs, TNFα activated various downstream signaling pathways, including activation of the PI3K (26) pathway and the MAPK family members JNK, ERK1/2, and p38 (27). In our study performed using HUVECs, TNFα efficiently phosphoryl-
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PD98059 in TNFα-treated HUVECs dramatically down-regulated VCAM-1 expression. These data provide indirect evidence of a role for the ERK pathway in the regulatory action of Lnk in ECs.

Moreover, our results have demonstrated that Lnk expression triggered persistent activation of the PI3K pathway in both resting and TNFα-treated ECs. PI3K activation was reflected by high and permanent phosphorylation of the downstream kinase Akt. In ECs, activation of PI3K gives rise to activation, through phosphorylation, of several downstream target proteins (28). Our findings indicate that Lnk-mediated activation of the PI3K/Akt pathway leads to eNOS phosphorylation and up-regulates HO-1 expression. Here we have shown that inhibition of PI3K, using a pharmacological inhibitor LY294002, totally abrogated HO-1 regulation and expression. Our results confirm a previous study by Salinas et al. (30) by showing a role for the PI3K in HO-1 regulation. HO-1 was shown to be phosphorylated by Akt, and an increase in in vitro HO-1 activity of cells expressing active Akt has been observed (30). In this study, inhibition of PI3K totally abrogated HO-1 regulation, whereas inhibition of PI3K with wortmannin had no significant effect on TNF-induced VCAM-1 expression. Several studies have provided evidence that HO-1 functions as an intrinsic defense system in cardiovascular disorders. Cytotoxic effects of HO-1 have been observed recently in many types of cells, such as cardiomyocytes, hepatocytes, and fibroblasts, as well as in vascular ECs (31, 32). In ECs, HO-1 expression correlated with down-regulation of VCAM-1 and E-selectin but not ICAM-1 in response to TNFα (32). However, although HO-1 is a potent anti-inflammatory molecule able to modulate cellular adhesion molecule expression, HO-1 seems not to be generally involved in the anti-inflammatory function of Lnk. Nevertheless, Akt, as well as HO-1 and l-nitro arginine methyl ester, are well described mediators of EC survival and proliferation (28, 33). Thus, the functional consequences of the positive regulation of PI3K, eNOS, and HO-1 mediated by Lnk have to be further determined. It is notable that, consistent with our data, Madge and Pober (26) have shown that the Akt pathway does not contribute to NFκB activation and adhesion molecule expression in human EC.

MAPKs include the extracellular signal-regulated kinases ERK1 and ERK2, the c-Jun NH2-terminal kinases JNK1, JNK2, and JNK 3, and the four p38 enzymes p38α, p38β, p38γ, and p38δ (34). ERK1/2 is known to be involved in the regulation of cell proliferation and apoptosis. In this study, we have shown that Lnk is an effective inhibitor of ERK1/2 activation in ECs, as previously reported in other cell types (14). Given that ERK1/2 activation can act in proinflammatory pathways (35), ERK1/2 inhibition may account for the decreased inflammatory processes observed in ECs expressing Lnk. Furthermore, the ability of the MEK1/2 inhibitor PD98059 to abrogate VCAM-1 expression ascribes functional significance to ERK1/2 activation in the pathway leading to EC activation and inflammation. ERK1/2 is phosphorylated and subsequently stimulates the binding of a variety of transcription factors, including AP-1, SP-1, and NFκB (36). Cytokine-dependent VCAM-1 induction is regulated at the gene level by the activity of transcription factors including NFκB, AP-1,
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SP-1, IRF-1, and GATA (37). Therefore, inhibition of ERK by Lnk could lead to reduced activity of transcription factors controlling VCAM-1 expression. Alternatively, Jiang et al. (38) recently demonstrated that inhibition of ERK did not affect interleukin-1β-induced IkBα phosphorylation and degradation but attenuated IkBβ degradation in smooth muscle cells. These results suggest a novel functional role for ERK as an important temporal regulator of NFκB activation and NFκB-dependent gene expression.

Consistent with a role for Lnk in ERK regulation, it has been proposed that the elevated proliferation of Lnk−/− pre-B cells observed in Lnk knock-out mice was because of an inability to regulate ERK activation properly in the absence of Lnk expression (14). Furthermore, in hematopoietic progenitors, Lnk selectively inhibits c-kit-mediated proliferation by inhibiting tyrosine phosphorylation of Gab2 and activation of the MAPK cascade (16). Moreover, Lnk also suppresses stem cell factor-induced ERK activation (18).

Our data also provide evidence for a regulatory loop between PI3K and ERK pathways. The PI3K pathway has been shown to interact with the ERK1/2 pathway in some cells (39, 40), including in human ECs (26), and several possible molecular intermediates have been shown to facilitate cross-talk between the PI3K- and ERK-signaling pathways (33). The potential role of Lnk at the signaling intersection between PI3K and ERK (MAPK) and the cross-talk between the two kinases needs further investigation and may be of great significance in the understanding of vascular biology.

Lnk, with APS and SH2-B, belongs to a newly discovered family of protein adaptors. These adaptors do not possess a kinase domain but contain several protein-protein interaction domains, including a proline-rich N terminus, a pleckstrin homology domain, a SH2 domain, and a conserved tyrosine near the C terminus (15). Thus, a key unresolved issue is the exact mechanism by which Lnk activates PI3K and inhibits ERK signal transduction pathways. In particular, the role of the different domains of Lnk required to control TNF signaling remains to be explored using deletion mutants for the tyrosine at the C terminus and the SH2 and pleckstrin homology domains of human Lnk.

Interestingly, in mice, Lnk was found to be expressed predominantly in the endothelial cells lining the dorsal aorta at embryonic day 11.5 (18). In vitro differentiation of embryonic stem cells gives rise to a population of definitive hematopoietic cells via endothelial precursor cells. Overexpression of Lnk in the primary culture of the AGM region at embryonic day 11.5 suppressed the emergence of CD45+ hematopoietic cells (18). Among mice nullizygous for the Lnk family members Lnk, APS, and SH2-B, Lnk-deficient mice exhibit the most profound alteration in hematopoiesis, including abnormal expansion of immature B cells (15), enhanced bone marrow repopulating activity (16), and elevated numbers of megakaryocytes (23) and erythroid progenitors (24). Taken together, these data suggest that Lnk may be a key player in EC differentiation, activation, and dysfunction. To conclude, these data support the evolving concept that Lnk is a key component of biomechanical signal transduction and establish Lnk as a potentially novel regulator of TNFα signaling and inflammation in the endothelium.

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