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TNF-α Induces Lnk Expression Through PI3K-Dependent Signaling Pathway in Human Umbilical Vein Endothelial Cells

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Background. **A better understanding of activation process of endothelial cells (ECs) might reveal new ways of controlling inflammation. Adaptor proteins play crucial roles in ECs activation. Lnk is a newly discovered adaptor protein that has been proposed as a negative regulator of cytokine signaling. While limited information is available about Lnk in human ECs. This study was conducted to investigate the effect of TNF-α on Lnk expression in ECs and to identify the signal transduction pathway that is associated with Lnk regulation.**

Materials and methods. **Primary human umbilical vein endothelial cells (HUVECs) were cultured with designated doses of TNF-α and harvested at designated time points. Then Lnk mRNA and protein were detected using real-time polymerase chain reaction, immunoprecipitation and Western blot analysis, respectively.**

Results. **The data demonstrated that Lnk mRNA and protein expression are induced significantly ($P < 0.05$) by TNF-α in a dose- and time-dependent manner. This inductive effect was abolished while phosphatidylinositol 3-kinase (PI3K) pathway was blocked by the PI3K inhibitor LY294002 and Wortmannin.**

Conclusion. **These results suggest that TNF-α induces Lnk expression through PI3K-dependent signaling pathway in HUVEC. This may indicate a role for this new adaptor protein in the regulation of TNF-α-induced ECs activation.**

Key Words: **endothelial cells; Lnk; adaptor protein; TNF-α; PI3K.**

INTRODUCTION

Endothelial cells (ECs) activation is the key event associated with acute and chronic inflammation [1]. Activated ECs express new proteins and secrete chemokines, regulating the inflammatory response. A better understanding of activation process of ECs might reveal new ways of controlling inflammation. In the activation of ECs, the formation of multimolecular complexes, which are initiated by adaptor proteins in many cases, is a crucial step [2].

Tumor necrosis factor-alpha (TNF-α) is an important proinflammatory cytokine, the primary targets of which include vascular endothelial cells [3]. Increasingly, the intracellular pathways that are activated in response to TNF-α have been elucidated in ECs. Adaptor proteins were found to play crucial roles in ECs activation. The binding of TNF to its cell surface receptors causes recruitment of specific adapter signaling proteins to intracellular domains of receptors, resulting in new protein synthesis and new gene transcription [4].

Lnk, a member of an adaptor protein family that also contains APS and SH2-B, is implicated in cytokine receptor signaling [5–8]. Lnk was originally cloned from a rat lymph node cDNA library and described as a 38 kDa adaptor protein participating in T lymphocytes signaling [8]. Mice nullizygous for Lnk revealed an essential role for Lnk in B cell lymphopoiesis and early hematopoiesis [9–11]. Further study showed that Lnk mRNA was up-regulated by TNF-α in primary porcine endothelial cells, indicating a role of Lnk in inflammation [12]. However, the molecular mechanism for regulation of Lnk expression in ECs has not been reported.

Considering the fact that TNF-α signaling responses vary widely among different cell types, we wonder if Lnk expression levels are also modified by TNF in human ECs. This study was conducted to investigate the effects of TNF on the expression of Lnk in primary human umbilical vein endothelial cells (HUVECs).

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Furthermore, we identified the signal transduction pathway that was associated with Lnk regulation.

MATERIALS AND METHODS

Cell Culture and Activation

Primary cryopreserved, pooled human umbilical vein endothelial cells (HUVEC, No. C-003-5C; first-passage) were obtained from Cascade Biologics (Portland, OR). The cells have the characteristics of vascular endothelial cells in terms of cobblestone appearance at confluence and von Willebrand factor expression. After thawing, cells were seeded in 75 cm^2 cell culture flasks and cultured according to the supplier's recommendations in medium 200. Medium 200 was supplemented with low-serum-growth supplement (20 μL/mL) (Cascade Biologics) in the absence of antibiotics. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The primary cells were used between passages 3 and 4 in this study.

For time course study, HUVECs were treated with 20 ng/mL TNF-α (Sigma, Saint Louis, MO) for 4, 8, 16, or 24 h. For dose-dependent study, HUVECs were treated with 5, 10, 20, or 40 ng/mL TNF-α for 16 h. Such TNF-α concentrations are in the range of concentrations found in the case of severe sepsis, and are able to activate ECs [13–15]. Control cultures received PBS alone. To identify the signal transduction pathway, cells were pretreated for 12 h with 10 μM LY294002 (Sigma) or 50 nM Wortmannin (Sigma), the two specific inhibitors of phosphatidylinositol 3-kinase (PI3K), then cultured with 20 ng/mL TNF-α for 16 h. For the mRNA and protein detection, cells were harvested with 0.25% trypsin and 0.02% EDTA (Sigma).

RNA Isolation and cDNA Synthesis

Cells were harvested by trypsinization and total RNA was isolated using RNeasy reagents (Qiagen, Chatsworth, CA) according to the manufacturer's protocol and quantitated by spectrophotometry. cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μL. Final concentration of reaction components: 5 mM $MgCl_2$, 500 ng oligo (dT) primer, 1U/μL RNase-Inhibitor, 15 U/μL AMV reverse transcriptase, 1× Buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTP, 50 ng/μL total RNA. Reactions were performed using the Reverse Transcription System (Promega, Madison, WI) under the following conditions: 42°C for 15 min, 95°C for 5 min, 4°C for 5 min. Samples were stored at −20°C until use.

Real-Time Quantitative PCR

The cellular levels of Lnk gene expression were quantified by real-time quantitative Taqman PCR using an ABI PRISM 7700 (Perkin–Elmer, Wellesley, MA). Specific primers and dual-labeled fluorescent probes were designed to detect Lnk mRNA using the Primer Express primer design program v1.01 (Perkin–Elmer). The constitutively expressed β-actin was used as an internal control. Probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5′ end and the quencher N,N,N,N′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end. The primer and probe sequences were as follows: Lnk forward primer 5′-TCC AGC TAC GTG GTA GTC GTC TC-3′, Lnk reverse primer 5′-GAA GAA CTA AGG TGG GGA AGG C-3′ and Lnk probe 5′-(FAM)-CCA CCA GGT TCC TGC AAC ACG GTC-(TAMRA)-3′. β-actin forward primer 5′-AGA AGG AGA TCA CTG CCC TG-3′, β-actin reverse primer 5′-ACA TCT GCT GGA AGG TGG AC-3′ and β-actin probe 5′-(FAM)-AAT GAT CTT GAT CTT CAT TGT G-(TAMRA)-3′.

Samples were amplified in a final volume of 25 μL. Primers were used at a concentration of 900 nM, probes at a concentration of 250 nM. Beta-actin was amplified in separate reactions. The cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 30 s, and 60°C for 30 s.

Data were analyzed using the cycle threshold (C_T) comparative method [16]. The PCR efficiency of the target (Lnk mRNA) and of the control (β-actin) was equal to the cDNA concentrations used in these experiments. The amount of mRNA levels was given by $(1 + E_{\text{PCR efficiency}})^{-\Delta\Delta C_T}$, where $-\Delta\Delta C_T = [C_T \text{ target} - C_T\ \beta\text{-actin}]_{\text{TNF-A}} - [C_T \text{ target} - C_T \text{ GAPDH}]_{\text{control}}$ and C_T = PCR cycle at which the amplification plot crosses the baseline threshold. The fold increase in target mRNA relative to control is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_{T+s}$ and $\Delta\Delta C_{T-s}$, where s = standard deviation of the $\Delta\Delta C_T$ value.

Immunoprecipitation

Cells were harvested and lysed in IP buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1 ug/mL leupeptin, 1 mM Deoxycholic acid and 1 mM EDTA) for 30 min at 4°C. The lysis product was centrifuged at 4°C, 20,000 × g for 30 min. The supernatant was collected and the protein content was measured by Modified lowry protein assay kit (Pierce, Rockford, CT). Equal amounts of proteins of 1 mg were incubated with 2.0 μg goat-anti-Lnk antibodies (Serotec, Oxford, UK) overnight at 4°C. The protein-antibodies complexes were collected on protein A-Sepharose beads. The beads were washed three times with IP buffer and prepared for Western blot.

Western Blot Analysis

Proteins were boiled for 5 min in loading buffer (1M Tris-HCl, 10% β-mercapt-oethanol, 10% SDS, 20% Glycerol) and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were transferred to a polyvinylidine difluoride (Millipore, Bellerica, MA) membrane. The membranes were blocked for 30 min with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST). The samples were incubated with primary antibody Goat anti-Lnk (1:400) (Serotec) overnight at 4°C. The membranes were washed three times in TBST for 5 min each and then blotted for 2 h with horseradish peroxidase-conjugated secondary antibody Rabbit anti-Goat IgG (1: 500)(Santa Cruz, Santa Cruz, CA) at room temperature. The membranes were then washed twice with TBST followed by an additional wash of TBS. The immunoreactive bands were visualized using ECL and Western Blotting Detection System (Santa Cruz). The protein mass was compared after quantifying the intensity of protein bands by Quantity one software (Bio-Rad). Experiment was repeated for three times.

Statistical Analysis

Data are expressed as mean ± SD. Statistical significance between two groups was tested using the unpaired Student's t-test. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

RESULTS

TNF-α Up-Regulates Lnk Expression Levels in HUVECs

To investigate the modulation of the Lnk expression by TNF-α, cells cultured with designated doses of TNF-α were harvested at designated time points. Real-time PCR showed that the Lnk expression curve appeared much later than the β-actin. A time course experiment revealed that the stimulatory effect of TNF-α on Lnk mRNA could be seen already after 4 h and was still present after 24 h of incubation with TNF-α. Maximal up-regulation of Lnk was at 16 h